(54) Title: PRODUCTION OF ANTI-SELF ANTIBODIES FROM ANTIBODY SEGMENT REPERTOIRES AND DISPLAYED ON PHAGE

(57) Abstract

Methods are disclosed for the production of anti-self antibodies and antibody fragments, being antibodies or fragments of a particular species of mammal which bind self-antigens of that species. Methods comprise providing a library of replicable genetic display packages (rgdps), such as filamentous phage, each rgdp displaying at its surface a member of a specific binding pair which is an antibody or antibody fragment, and each rgdp containing nucleic acid sequence derived from a species of mammal. The nucleic acid sequence in each rgdp encodes a polypeptide chain which is a component part of the sbp member displayed at the surface of that rgdp. Anti-self antibody fragments are selected by binding with a self antigen from the said species of mammal. The displayed antibody fragments may be scFv, Fd, Fab or any other fragment which has the capability of binding antigen. Nucleic acid libraries used may be derived from a rearranged V-gene sequences of unimmunised mammal. Synthetic or artificial libraries are described and shown to be useful.

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PRODUCTION OF ANTI-SELF ANTIBODIES FROM ANTIBODY SEGMENT REPERTOIRES AND DISPLAYED ON PHAGE

This invention relates to the isolation of antibody molecules directed against self antigens, e.g. human

5   antibodies directed against human self antigens. Phage display technology for selection of antibody molecules was described in WO92/01047, PCT/GB92/00883, PCT/GB92/01755 and GB9206372.6. The applicants have realised that antibodies directed against self antigens

10   can be isolated using phage display technology.

Human antiself antibodies are of particular value for in vivo therapeutic and diagnostic purposes, since they avoid the problems arising from the antigenicity of foreign, e.g. mouse antibodies. The most useful human

15   antibodies for therapy are those directed against cell surface molecules, such as receptors, adhesins and integrins, and those directed against circulating biological effector molecules, such as hormones, growth factors and cytokines. It has been extremely difficult to

20   obtain human antibodies against such self antigens. This invention provides a powerful way of obtaining such antibodies.

It is a demanding task to isolate an antibody fragment with specificity against self antigen. Animals

25   do not normally produce antibodies to self antigens, a phenomenon called tolerance (G.J.Nossal Science 245 147-153, 1989). Autoimmune diseases may result from a breakdown in tolerance. In general, vaccination with a self antigen does not result in production of circulating

30   antibodies. It is therefore difficult to raise antibodies to self antigens, particularly in humans. It is possible to raise antibodies that recognise human antigens in an animal such as a mouse, especially if the human antigen is not too closely related to any

35   equivalent in the animal. If a human antibody is then required it is necessary to 'humanise' the antibody, e.g. by CDR grafting (patent GB2188638B).

Phage antibody technology as described in (WO92/01047) offers the ability to isolate such human

40   antibodies directly. In this application, we demonstrate

for the first time that antibodies against self-antigens can be isolated from phage libraries derived from, for example, nonimmunised sources and from libraries prepared by synthetic recombination of V-gene sequences,
5   preferably recombination of VH with, DH and JH, and VL with JL sequences. These antibodies are specific for their antigen. This application shows that single libraries derived in this manner can act as a source of both foreign and self antigens and opens up the prospect
10 of a large, universal library to isolate antibodies to any antigen.

It was disclosed in patent application WO92/01047 that antibody fragments can be displayed on the surface of bacteriophage and that they will bind antigen.
15 Antibody fragments can be directly selected using this characteristic. This ability to isolate antibody fragments (Fab, Fv, scFv and VH) using their display on the surface of filamentous bacteriophage has opened up the prospect of the isolation of antibody specificities
20 (i.e. antibodies directed against a particular antigen) that were difficult or impossible to isolate previously. In particular WO92/01047 demonstrates that antibody specificities can be isolated from a human who has not been specifically immunised ('unimmunised'), even
25 specificities for antigens such as 2-phenyl-5-oxazolone to which humans will not normally be exposed.

In embodiments of this invention, natural or synthetic antibody repertoires derived from a species of mammal, such as human, mouse, rat, sheep, pig, goat,
30 horse or other, are displayed on the surface of a replicable genetic display package (rgdp) and the binding specificity for self is selected by binding to self antigen. In this process, the V gene repertoires are derived from V genes rearranged _in vitro_ or _in vivo_ and
35 or by mutation of (a) rearranged V gene(s). A key feature of the V gene repertoires is that they are extremely diverse in sequence, usually in excess of 10^6 different members. Indeed it is possible that a sufficiently large

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library may provide a source of specificities directed
against any self antigen. The V-gene repertoires are
cloned into the rgdp (for example a filamentous phage
vector) such that antibody repertoires are displayed on
5   the surface of the rgdp. The rgdps encoding rare antibody
specificities binding to antiself, may be selected by
virtue of binding to the self antigen. The antibody
repertoires may be cloned in a single replicon or a dual
replicon format as described in WO92/01047 and
10   PCT/GB92/00883.

The V genes may be cloned into the genetic material
of the rgdp, and expressed as single domains, for example
single heavy chain variable domains, so called single
domain ligands or "dAbs" (see WO90/01544), or as
15   associated antibody heavy and light chain variable
domains.

The two domains could be displayed as separate
polypeptide chains (linked as in Fab fragments through
non-covalent association of domains and/or disulphide
20   bonds), or as part of the same chain (single chain Fv
fragments where the two domains are contained within the
same polypeptide chain).

In WO92/01047 and examples 1 to 8 of this
application we have used fusion of antibody fragments to
25   gene 3 protein of filamentous bacteriophage for display
and selection of antibody fragments. An alternative
approach would be to fuse antibody fragments to gene 8
protein or other surface molecules of filamentous
bacteriophage.

30   Isolation of human antibodies directed against human
antigens is a demanding task. There are only a limited
number of human antigens against which circulating human
antibodies are naturally found. Antibodies are present
directed against non-self antigens of human origin.
35   Antibodies directed against human blood group B have been
isolated from a phage display library prepared from
subjects of blood group O (J.D. Marks et al, J. Mol.
Biol. 222 581-597, 1991), which recognise the blood group

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B antigen as foreign.

This invention is concerned with a general method for the isolation of antibodies directed against self antigens which are specific for the antigen concerned.

5 Many patients show significant concentrations of circulating autoantibodies. It is estimated that 10 to 30% of B lymphocytes in normal, healthy individuals are engaged in making autoantibodies (I.R. Cohen and A. Cooke Immunol. Today 7 363-364, 1986). However, the 'natural

10 autoantibodies' produced do not lend themselves to therapeutic use as they are often IgM, low affinity and polyreactive (P. Casali and A.L. Notkins Ann. Rev. Immunol. 7 515-531, 1989; S. Avrameas Immunol. Today 12 154-159). An immune response against self can arise in

15 autoimmune disease or after infections and a few monoclonal antibodies directed against self antigens have been isolated from patients with autoimmune disease (K. James & G.T. Bell J. Immunol. Methods 100 5-40, 1987). These autoantibodies are frequently specific, but may

20 bind to only a limited range of epitopes on the antigen (M. Bouanani et al Arthritis Rheum. 34 1585-1593, 1991).

The preparation of V gene libraries derived from the mRNA of plasma cells secreting IgG (or IgM) antibody may thus lead to the isolation of antibody fragments derived

25 from autoantibodies. For instance, anti-self antibodies might be isolated from patients with autoimmune diseases, for example anti-acetylcholine receptor antibodies would be expected to be isolated from antibody repertoires made from the IgG mRNA of myasthenia gravis patients. For

30 example, an antibody fragment specific for human thyroid peroxidase has been isolated from a bacteriophage lambda library from a patient with thyroid autoimmune disease (S.Portolano et al Biochem. Biophys. Res. Commun. 179 372-377, 1991). This however required extensive

35 screening of 200,000 plaques to obtain one clone. In addition, this library was derived from thyroid tissue, a procedure not readily applicable in most instances.

In contrast, the power of selection available using

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the phage system, demonstrated in WO92/01047 allows the
ready isolation of autoantibodies from the IgM mRNA of
peripheral blood lymphocytes of a donor without disease.
We show in example 2 that antibodies binding to human
thyroglobulin (which can be found in the sera of people
with or without symptomatic autoimmune disease), can be
isolated from phage repertoires prepared from unimmunised
humans. One would not expect necessarily to be able to
obtain antibodies to human thyroglobulin by immunising a
human with human thyroglobulin, notwithstanding the
presence of thyroglobulin autoantibodies in many people.
Autoantibodies against thyroglobulin in normal sera have
been reported often to have a high degree of
polyreactivity (S.Avrameas, 1991 supra). In contrast,
those which are isolated using a method according to the
present invention involving phage antibody technology,
see example 2 for instance, are specific for
thyroglobulin.

In this application, we also demonstrate that even
antibodies against human tumour necrosis factor-α can be
isolated as described in example 1 from the same library
as the antibodies directed against thyroglobulin. Many
self antigens do not have detectable associated
circulating autoantibodies. Further, example 3 shows the
isolation of antibodies against the self antigens mucin,
carcinoembryonic antigen (CEA) and CD4, antibodies to
which have not been reported in normal sera. Moreover,
these antibodies are specific, whereas there is often a
high degree of polyreactivity in natural autoantibodies
which can sometimes be found. The vast majority of self
antigens do not have detectable associated circulating
autoantibodies. Thus the isolation of antiself
antibodies as described in this invention opens the
prospect of the direct isolation of human antibodies
binding to human antigens for a number of purposes such
as antibodies which bind to circulating hormones to
block, modify or potentiate their action or antibodies
that bind to cell surface antigen for imaging or killing

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for example of cancer cells.

The origin of the V genes that contribute to anti-self antibodies isolated from phage display libraries is not clear. Tolerance to self antigens by the immune system (preventing the generation of antibodies directed against them) is mediated by either clonal deletion or functional inactivation (anergy) of self-reactive B lymphocytes (D.A.Nemazee & K.Burki Nature 337 562-566, 1989; C.C.Goodnow et al Nature 334 676-682, 1988; S.B.Hartley et al Nature 353 765-769, 1991; D.M.Russell et al Nature 354 308-311, 1991). In either case little circulating anti-self antibody is detectable for most antigens. However, in the case of anergy, functionally inactivated self-reactive cells from the B cell lineage persist in peripheral lymphoid organs leading to B cells in circulation. These rare lymphocytes with anti-self specificity may provide heavy or light chain partners (or even both) for phage antibodies with anti-self specificities. Alternatively, such anti-self specificities may arise from the combination in the library of a VH domain with a VL domain to give a specificity that is normally deleted if it occurs in nature. For this reason, combinatorial libraries and 'chain-shuffled' libraries such as described in patent applications WO92/01047 may be a particularly rich source of anti-self antibodies. A selection procedure of great power, such as that provided by phage antibodies, is required to obtain such rare anti-self antibodies.

The degree of somatic mutation observed in antiself antibody fragments isolated by phage technology in this application indicates that some have germ line sequences and have therefore arisen from virgin B cells. Other antibodies isolated by phage antibody technology in this application show somatic hypermutation indicating that the V genes have been stimulated by antigen, either a foreign cross reactive antigen or other foreign antigens. In both cases the antibody fragments isolated using phage

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technology will usually be a combination of VH and VL
domains not originally present in the B lymphocytes and
the power of phage technology, as described in this
application enables their isolation.

5   According to the present invention there is provided
a method of obtaining a member of a specific binding pair
(sbp member), which sbp member has an antigen binding
site with binding specificity for an antigen which is a
self antigen of a species of mammal, the method

10   comprising:

       (a)   providing a library of replicable genetic
       display packages (rgdps), each rgdp displaying at
       its surface an sbp member, and each rgdp containing
       nucleic acid with sequence derived from said species

15   of mammal and encoding a polypeptide chain which is
       a component part of the sbp member displayed at the
       surface of that rgdp;

       (b)   selecting, by binding with said self antigen,
       one or more sbp members with binding specificity for

20   said self antigen.

The polypeptide component part encoded by the
nucleic acid in each rgdp may be a VH or VL domain of an
antibody, or any part of an antibody which, either alone
or in combination with one or more other component parts,

25   forms an antibody fragment which is capable of binding an
antigen. Examples of polypeptide chains which may be
used as component parts of an sbp member as described
above therefore include, in addition to VH and VL
domains, $V_L C_L$, $V_H C_H 1$, scFv fragments, Fab fragments and

30   so on.

Each said sbp member displayed at the surface of an
rgdp may be an antibody fragment comprising a V_H domain
and a V_L domain.

Each antibody fragment may be a scFv fragment, a Fab

35   fragment, a Fv fragment consisting of the V_L and V_H
domain of a single arm of an antibody, a single domain
binding ligand consisting essentially of or comprising a
heavy-chain variable domain (Fd), or any other fragment

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which has the capability of binding an epitope or antigen.

The step of providing a library of rgdps may comprise:

5   combining (i) a first polypeptide chain component part of an sbp member fused to a component of a rgdp which thereby displays said first polypeptide chain component part or population thereof at the surface of rgdps on expression in a

10   recombinant host cell organism, or a population of such a first polypeptide chain component part fused to a said component of a rgdp, with (ii) a second polypeptide chain component part of an sbp member or a population of such a second polypeptide chain

15   component part, to form a library of sbp members displayed at the surface of rgdps;

           at least one of said first or second polypeptide chain component part or populations thereof being encoded by nucleic acid which is

20   capable of being packaged using said component of an rgdp.

The step of providing a library of rgdp may comprise:

           expressing in a recombinant host organism a

25   first polypeptide chain component part of an sbp member or a population of such a first polypeptide chain component part, fused to a component of an rgdp which thereby displays said polypeptide chain component part at the surface of rgdps;

30   combining said first polypeptide chain component part or population with a second polypeptide chain component part of an sbp member or a population of such a second polypeptide chain component part, to form a library of rgdps each

35   displaying an sbp member at its surface, at least one of said polypeptide chain component parts being expressed from nucleic acid which is capable of being packaged using said component of an rgdp.

Where the sbp member is an Fab fragment the first and second polypeptide chain component part may be a polypeptide consisting of a V_L and a C_L domain, and the second polypeptide chain component part a polypeptide consisting of a V_H and a C_H1 domain.

The combining of first and second polypeptide chain component parts or populations thereof may be at the nucleic acid level with expression vectors each having introduced therein a sequence encoding a first component part and a sequence encoding a sequence component part. On the other hand, the combining may be at the polypeptide level with first component parts not being expressed from the same vectors as second component parts. Indeed, one or other of the first and second component parts may be provided as a soluble library. Details of various formats which may be employed are given in WO92/01047 and PCT/GB92/00883.

The step of providing a library may comprise:

combining (i) nucleic acid which encodes a first polypeptide chain component of an sbp member fused to a component of a rgdp or a population of such a first polypeptide chain component part fused to a component of a rgdp, with (ii) nucleic acid encoding a second polypeptide chain component part of an sbp member or a population thereof, to form a library of nucleic acid, nucleic acid of said library being capable of being packaged using said component of an rgdp;

expressing in a recombinant host organism said first polypeptide chain component part fused to a component of a rgdp or population thereof and said second polypeptide chain component part of an sbp member or a population thereof, to produce a library of rgdps each displaying at its surface an sbp member and containing nucleic acid encoding a first and a second polypeptide chain component part of the sbp member displayed at its surface.

Readers are urged to consult WO92/01047, in

particular, if further details of any method described herein are desired.

In one embodiment of the present invention both first and second polypeptide chain component parts or populations thereof are expressed from nucleic acid capable of being packaged using said component of an rgdp. This might be when the component parts together form a Fab fragment or, more usually, when each said sbp member displayed at the surface of an rgdp is an scFv antibody fragment.

In one embodiment, each said second polypeptide chain component part or population thereof may be expressed from nucleic acid separate from nucleic acid from which said first polypeptide chain component part or population thereof is expressed. The nucleic acid encoding the first polypeptide chain component part may be on the same expression vector as the nucleic acid encoding the second polypeptide chain component part, but separate from it so that, for example, Fab fragments are produced. Alternatively, the nucleic acid encoding the first polypeptide chain component part may be on a different expression vector from the nucleic acid which encodes a second polypeptide chain component part. Where a first and second polypeptide chain component part are both encoded on the same expression vector then they may be expressed as scFv fragments, where a VH domain is joined to a VL domain by a polypeptide linker, so that each scFv is a single polypeptide chain.

Each sbp member displayed at the surface of an rgdp is an Fab antibody fragment.

The nucleic acid may be derived from, e.g. rearranged V genes of, an unimmunised mammal, for example a mouse, rat, rabbit, sheep, pig, horse, goat, dog or human. Preferably the species of mammal is human, since it is most difficult to obtain antibodies which recognise (i.e. bind specifically) human self antigens.

The nucleic acid may be derived from a library prepared by artificial or synthetic recombination of V-

gene segments, which may be germ-line V-gene sequences.
The library may be totally synthetic.

Sbp members selected in (b) displayed at the surface
of rgdps may be selected or screened to provide an
individual sbp member or a mixed population of said sbp
members associated in their respective rgdps with nucleic
acid encoding said sbp member or a polypeptide chain
thereof. Rgdp phage displaying sbp members selected in
(b) may be grown to increase their numbers before any
subsequent further selection or screening. Nucleic acid
which encodes a selected or screened sbp member and which
is derived from an rgdp which displays at its surface a
selected or screened sbp member may be used to express an
sbp member or a fragment of derivative thereof in a
recombinant host organism.

The present invention encompasses any method wherein
nucleic acid from one or more rgdps selected from the
library by binding with a self antigen is taken and used
to provide encoding nucleic acid in a further method
(according to any embodiment of the present invention or
not) to obtain an individual sbp member or a mixed
population of sbp members, or encoding nucleic acid
therefor.

The expression end product, selected sbp member, may
be modified to produce a derivative thereof.

The expression end product or derivative thereof may
be used to prepare a therapeutic or prophylactic
medicament or a diagnostic product.

The present invention also encompasses antibody
fragments, derivatives thereof, including whole
antibodies and fusions with enzymes, obtained using any
method described herein according to the present
invention.

According to an aspect of the present invention,
there is provided use, in any method according to any
embodiment of the present invention described herein, of
a kit comprising a library of vectors each comprising
nucleic acid which is capable of being packaged in rgdps

and which encodes a polypeptide chain component part of an antibody for display at the surface of rgdps.

There is also provided by the present invention use, in any method according to any embodiment of the present invention described herein, of a kit comprising a library of rgdps each containing nucleic acid encoding at least one polypeptide chain component part of an antibody.

The present invention provides generally a method for producing a replicable genetic display package (rgdps) or population of such rgdps, which method comprises the steps of:

(a) inserting a nucleotide sequence encoding a binding molecule which is a member of a specific binding pair and an anti-self antibody, within a viral genome;

(b) culturing the virus containing said nucleotide sequence so that said binding molecule is expressed and displayed by the virus at its surface.

The present invention also provides a method for selecting a rgdp specific for a particular self-antigen epitope which comprises producing a population of such rgdps and the additional step of selecting for said binding molecule which is an anti-self antibody by contacting the population with said epitope so that individual rgdps with the desired specificity may bind to said epitope. The method may comprise one or more of the additional steps of: (i) separating any bound rgdps from the epitope; (ii) recovering any separated rgdps and (iii) using the inserted nucleotide sequences from any separated rgdps in a recombinant system to produce the binding molecule separate from the virus. The selection step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus in which said encoding nucleic acid is contained.

The present invention also provides a method of producing a multimeric member of a specific binding pair (sbp) which is an anti-self antibody, which method

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comprises:

expressing in a recombinant host organism a first
polypeptide chain of said sbp member or a genetically
diverse population of said sbp member fused to a
5   component of a secreted replicable genetic display
package (rgdp) which thereby displays said polypeptide at
the surface of the package, and expressing in a
recombinant host organism a second polypeptide chain of
said multimer and causing or allowing the polypeptide
10   chains come together to form said multimer as part of
said rgdp at least one of said polypeptide chains being
expressed from nucleic acid that is capable of being
packaged using said component therefor, whereby the
genetic material of each said rgdp encodes a said
15   polypeptide chain.

       Both said chains may be expressed in the same host
organism.

       The first and second chains of said multimer may be
expressed as separate chains from a single vector
20   containing their respective nucleic acid.

       At least one of said polypeptide chains (or
polypeptide chain component parts) may be expressed from
a phage vector.

       At least one of said polypeptide chains may be
25   expressed from a phagemid vector, the method including
using a helper phage, or a plasmid expressing
complementing phage genes, to help package said phagemid
genome, and said component of the rgdp is a capsid
protein therefor. The capsid protein may be absent,
30   defective or conditionally defective in the helper phage.

       The method may comprise introducing a vector capable
of expressing said first polypeptide chain, into a host
organism which expresses said second polypeptide chain in
free form, or introducing a vector capable of expressing
35   said second polypeptide in free form into a host organism
which expresses said first polypeptide chain.

       Each of the polypeptide chain may be expressed from
nucleic acid which is capable of being packaged as a rgdp

using said component fusion product, whereby encoding nucleic acid for both said polypeptide chains are packaged in respective rgdps.

The fusions may be expressed in the absence of the rgdp display component, perhaps capsid, expressed in wild-type form.

The capsid protein may be absent, defective or conditionally defective in the helper phage.

The host cell may be a mutator strain which introduces genetic diversity into the sbp member nucleic acid.

The rgdp may be a bacteriophage, the host a bacterium, and said component of the rgdp a capsid protein for the bacterophage. The phage may be a filamentous phage. The phage may be selected from the class I phages fd, M13, f1, If1, lke, ZJ/Z, Ff and the class II phages Xf, Pf1 and Pf3. The phage may be fd or a derivative of fd. The derivative may be tetracycline resistant. The said sbp member or polypeptide chain thereof may be expressed as a fusion with the gene III capsid protein of phage fd or its counterpart in another filamentous phage. The sbp member or polypeptide chain thereof may be inserted in the N-terminal region of the mature capsid protein downstream of a secretory leader peptide. The sequence may be inserted after amino acid +1 of the mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the nucleic acid to be inserted.

The host may be E.coli.

Nucleic acid encoding an sbp member polypeptide may be linked downstream to a viral capsid protein through a suppressible translational stop codon, so that under conditions where the stop is supressed fusion proteins are produced comprising sbp member polypeptide and viral capsid protein, while under non-supressing conditions free form sbp member polypeptides are produced.

Selection systems and assay formats are discussed

elsewhere in this text. In these systems and formats,
the gene sequence encoding the binding molecule (eg. the
antibody) of desired specificity is separated from a
general population of rgdps having a range of specifies,
5 by the fact of its binding to a specific target (eg the
antigen or epitope). Thus the rgdps formed by said
expression may be selected or screened to provide an
individual sbp member or a selected mixed population of
said sbp members associated in their respective rgdps
10 with nucleic acid encoding said sbp member or a
polypeptide chain thereof. The rgdps may be selected by
affinity with a member complementary to said sbp member.

   Any rgdps bound to said second member may be
recovered by washing with an eluant. The washing
15 conditions may be varied in order to obtain rgdps with
different binding affinities for said epitope.
Alternatively, to obtain eg high affinity rgdps, the
complementary member (eg an epitope) may be presented to
the population of rgdps (eg pAbs) already bound to a
20 binding member in which case pAbs with a higher affinity
for the epitope will displace the already bound binding
member. Thus the eluant may contain a molecule which
competes with said rgdp for binding to the complementary
sbp member. The rgdp may be applied to said
25 complementary sbp member in the presence of a molecule
which competes with said package for binding to said
complementary sbp member. Nucleic acid derived from a
selected or screened rgdp may be used to express said sbp
member or a fragment or derivative thereof in a
30 recombinant host organism. Nucleic acid from one or more
rgdps may be taken and used to provide encoding nucleic
acid in a further said method to obtain an individual sbp
member or a mixed population of sbp members, or encoding
nucleic acid therefor. The expression end product may be
35 modified to produce a derivative thereof.

   A preferred source for the generation of diverse
libraries from unimmunised humans is IgM mRNA. It is was
found in example 43 of WO92/01047 that antibody fragments

directed against turkey egg lysozyme and
2-phenyl-5-oxazolone were much more readily isolated from
a phage library derived from the IgM mRNA from
unimmunised human donors, than from one prepared from IgG

5   mRNA. Furthermore, no 2-phenyl-5-oxazolone binding
antibody fragments could be isolated from a library of
2000000 phage antibody clones prepared from IgGmRNA of
unimmunised mice (T.Clackson et al, Nature 352
624-628.1991). Examples 1 to 3 of this application show

10   the isolation of antibodies specific for self antigen
from the IgM library. Although in these samples,
antiself specificities have been selected as single chain
Fv fragments in a single replicon format, antibody
specificities could be selected as Fab fragments in a

15   single replicon format or in a dual combinatorial, dual
replicon format (Hoogenboom et al, 1991 supra) for
instance using recombination with the loxP system
(PCT/GB92/00883).

Phage libraries may be prepared which are enriched

20   for antibodies directed against self. B lymphocytes
express surface IgM and surface IgD before stimulation
with antigen but express little soluble IgM or IgD.
These unstimulated cells are more likely to contain
antibody genes with anti-self specificities. In

25   contrast, terminally differentiated plasma cells which
secrete soluble antibodies express little surface
immunoglobulin. The preparation of cDNA for phage
library preparation using primers which are specific for
surface IgM or surface IgD will produce a repertoire of

30   antibody genes enriched for the naive, unselected genes
encoding V domains. In B lymphocytes which have been
functionally silenced by exposure to self there are
greatly reduced levels of surface IgM but unchanged
levels of surface IgD (C.C.Goodnow et al. supra). Hence,

35   a primer specific for surface IgD may be particularly
suitable for isolation of anti-self antibodies.

However, as demonstrated in this application, IgM
mRNA from unselected peripheral blood lymphocytes is one

preferred source of V genes for antiself specificities.
Other sources of such anti-self antibodies may be fetal
mRNA or cord blood mRNA (P.M.Lydyard et al Scand J
Immunol 31 33-43, 1990).

5   There is the potential for making repertoires for
phage display using the original combination of VH and VL
domains by the use of PCR and linkage of the genes
encoding them within cells expressing these domains. The
principle of 'In cell PCR', where the original VH/VL

10   pairing is maintained, was demonstrated in PCT/GB92/01483
and described in Embleton et al in Nucleic Acids Res.,
20, 3831-3837, 1992. This may be particularly useful if
lymphocytes can be selected at a stage before the
deletion of clones expressing anti-self antibodies.

15   In one embodiment of this invention, V gene
sequences, or even libraries prepared by the synthetic
recombination of V, D and J segments may be used. These
act as a rich source of anti-self antibodies. In
examples 5 to 7, we demonstrate that anti-self

20   specificities against TNF, human anti-rhesus D antibody
(OAK3) and human thyroglobulin can be isolated from a
phage antibody library prepared by the synthetic joining
of V. D and J segments. The use of germ line V genes for
this purpose, as shown in examples 5 to 7, should be

25   valuable for the isolation of anti-self antibodies as
there is some evidence that B lymphocytes directed
against soluble self antigens are functionally silenced
and those directed against multivalent membrane bound
self antigen are eliminated (S.B.Hartley et al supra;

30   D.M.Russell et al, supra). Thus, the use of synthetic
libraries made by VH, DH, JH or VK,JK or VL,JL
recombination in vitro or its equivalent may be
particularly advantageous for isolation of antibodies
directed against multivalent membrane bound self

35   antigens.
           In examples 5 to 7 we have used synthetic VH CDR3
segments incorporating sequences of random bases at the
V-D-J joining region and linked them to germ line VH gene

segments. Other strategies may be used such as making each of the CDR loops of random sequence or making the CDR loops of known canonical structures (C. Chothia et al, Nature 342 877-893, 1989) and incorporating random sequence elements. The germ line nature of the V and J segments could be altered by incorporation of specific or random alterations to the sequence or by using somatically mutated V gene regions. The strategy used in examples 5 to 7 has the advantage that the loop structures of the V gene segments form only a limited number of distinct folds and combinations of folds (C. Chothia et al J. Mol. Biol. 227 779-817, 1992) and have presumably evolved for stability and to create a distribution and range of binding sites well suited to match the structure of antigens. Moreover, the framework regions and first two hypervariable loops of both heavy and light chains of the synthetic human antibodies are likely to be identical in many different individuals. Such synthetic human antibodies could be less immunogenic than entirely artificial structures.

A further but less preferred alternative to the above natural and synthetic phage display libraries would be to prepare random mutagenesis libraries displayed on phage, derived from one or a few human antibody molecules and selecting anti-self antigen specificities from these.

SELECTION

Individual rgdps eg pAbs expressing the desired specificity for an antigen, can be isolated from a library using the conventional screening techniques (e.g. as described in Harlow, E., and Lane, D., 1988, supra Gherardi, E et al. 1990. J. Immunol. meth. 126 p61-68).

The applicants have also devised selection techniques that are practicable because of the unique properties of rgdps. The general outline of some screening procedures is illustrated in Figure 5 using pAbs as an example type of rgdp.

The population/library of pAbs to be screened could

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be generated from immunised or other animals; or be
created in vitro by mutagenising pre-existing phage
antibodies (using techniques well-known in the art such
as oligonucleotide directed mutagenesis (Sambrook, J., et
5   al., 1989 Molecular Cloning a Laboratory Manual, Cold
Spring Harbor Laboratory Press) but are preferably
derived from unimmunised humans or artificial
recombination of human V segments, as described
elsewhere. This population can be screened in one or
10 more of the formats described below with reference to
Figure 5, to derive those individual pAbs whose antigen
binding properties are different from sample c.

Binding Elution
       Figure 5(i) shows antigen (ag) bound to a solid
15 surface (s) the solid surface (s) may be provided by a
petri dish, chromatography beads, magnetic beads and the
like. The population/library of pAbs is then passed over
the ag, and those individuals p that bind are retained
after washing, and optionally detected with detection
20 system d. A detection system based upon anti-fd antisera
may be used (see, for instance, Example 4 of WO92/01047).
If samples of bound population p are removed under
increasingly stringent conditions, the binding affinity
represented in each sample will increase. Conditions of
25 increased stringency can be obtained, for example, by
increasing the time of soaking or changing the pH of the
soak solution, etc.

Competition
       Referring to figure 5(ii) antigen ag can be bound to
30 a solid support s and bound to saturation by the original
binding molecule c. If a population of mutant pAb (or a
set of unrelated pAbs) is offered to the complex, only
those that have higher affinity for antigen ag than c
will bind. In most examples, only a minority of
35 population c will be displaced by individuals from
population p. If c is a traditional antibody molecule,
all bound material can be recovered and bound p recovered
by infecting suitable bacteria and/or by use of standard

techniques such as PCR.

An advantageous application is where ag is used as a receptor and c the corresponding ligand. The recovered bound population p is then related structurally to the receptor binding site/and or ligand. This type of specificity is known to be very useful in the pharmaceutical industry.

Another advantageous application is where ag is an antibody and c its antigen. The recovered bound population p is then an anti-idiotype antibody which have numerous uses in research and the diagnostic and pharmaceutical industries.

At present it is difficult to select directly for anti-idiotype antibodies. pAbs would give the ability to do this directly by binding pAb libraries (eg a naive library) to B cells (which express antibodies on their surface) and isolating those phage that bound well.

In some instances it may prove advantageous to pre-select population p. For example, in the anti-idiotype example above, p can be absorbed against a related antibody that does not bind the antigen.

However, if c is a pAb, then either or both c and p can advantageously be marked in some way to both distinguish and select for bound p over bound c. This marking can be physical, for example, by pre-labelling p with biotin; or more advantageously, genetic. For example, c can be marked with an EcoB restriction site, whilst p can be marked with an EcoK restriction site (see Carter, P. et al., 1985, Nucl. Acids Res. 13, 4431-4443). When bound p+c are eluted from the antigen and used to infect suitable bacteria, there is restriction (and thus no growth) of population c (i.e. EcoB restricting bacteria in this example). Any phage that grew, would be greatly enriched for those individuals from p with higher binding affinities. Alternatively, the genetic marking can be achieved by marking p with new sequences, which can be used to specifically amplify p from the mixture using PCR.

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Since the bound pAbs can be amplified using for example PCR or bacterial infection, it is also possible to rescue the desired specificity even when insufficient individuals are bound to allow detection via conventional

5   techniques.

The preferred method for selection of a phage displaying a protein molecule with a desired specificity or affinity will often be elution from an affinity matrix with a ligand. Thus, self antigen or fragments thereof

10   may be used to elute specific phage antibodies from self antigen bound to a matrix. Alternatively, the homologous antigen from a different species may be bound to a matrix, a phage antibody library bound, and phage antibodies specific for the self antigen may be eluted

15   using self antigen. For instance, a bovine antigen may be bound to the matrix, a human phage antibody library bound and human antigen used for elution. Antiself antibodies thus isolated will be specific for epitopes shared between the bovine and human antigens. A further

20   but less preferred alternative may be to bind the phage non-specifically to a column and elute with self antigen. For instance, if a Fab phage library is bound to an anti-Fab affinity column, it may be washed at a pH which does not elute non-specific phage and then washed with

25   solution which is the same except it contains self antigen, eluting by virtue of the higher affinity for the mobile phase of phage expressing antibodies against the self antigen.

For each of these formats elution with increasing

30   concentrations of ligand should elute phage displaying binding molecules of increasing affinity. However, when eg a pAb binds to its antigen with high affinity or avidity (or another protein to its binding partner) it may not be possible to elute the pAb from an affinity

35   matrix with molecule related to the antigen. Alternatively, there may be no suitable specific eluting molecule that can be prepared in sufficiently high concentration. In these cases it is necessary to use an

elution method which is not specific to eg the antigen-antibody complex. Some of the non-specific elution methods generally used reduce phage viability for instance, phage viability is reduced with time at pH12

5 (Rossomando, E.F. and Zinder N.D. J. Mol.Biol. 36 387-399 1968). There may be interactions between eg antibodies and affinity matrices which cannot be disrupted without completely removing phage infectivity. In these cases a method is required to elute phage which does not rely on

10 disruption of eg the antibody - antigen interaction. A method was therefore devised which allows elution of bound pAbs under mild conditions (reduction of a dithiol group with dithiothreitol) which do not disrupt phage structure (Example 47 of WO92/01047).

15 The method of mild elution uses binding of the phage antibody population to biotinylated antigen and binding to streptavidin magnetic beads. Following washing to remove non-binding phage, the phage antibody is eluted and used to infect cells to give a selected phage

20 antibody population. A disulphide bond between the biotin and the antigen molecule allows mild elution with dithiothreitol. A particularly advantageous way of performing this selection is to use biotinylated antigen in excess but at or below a concentration equivalent to

25 the desired dissociation constant for the antigen-antibody binding. This method is advantageous for the selection of high affinity antibodies (R.E. Hawkins, S.J. Russell and G. Winter J.Mol.Biol. 226 889-896, 1992). Antibodies may also be selected for slower off rates for

30 antigen selection as described in (R.E. Hawkins et al, 1992 supra). The concentration of biotinylated antigen may gradually be reduced to select higher affinity phage antibodies. As an alternative, the phage antibody may be in excess over biotinylated antigen in order that phage

35 antibodies compete for binding, in an analagous way to the competition of peptide phage to biotinylated antibody described by J.K. Scott & G.P. Smith (Science 249 386-390, 1990).

This elution procedure is just one example of an elution procedure under mild conditions. A particularly advantageous method would be to introduce a nucleotide sequence encoding amino acids constituting a recognition

5   site for cleavage by a highly specific protease between the foreign gene inserted, in this instance a gene for an antibody fragment, and the sequence of the remainder of gene III. Examples of such highly specific proteases are Factor X and thrombin. After binding of the phage to an

10   affinity matrix and elution to remove non-specific binding phage and weak binding phage, the strongly bound phage would be removed by washing the column with protease under conditions suitable for digestion at the cleavage site. This would cleave the antibody fragment

15   from the phage particle eluting the phage. These phage would be expected to be infective, since the only protease site should be the one specifically introduced. Strongly binding phage could then be recovered by infecting eg. E.coli TG1 cells.

20   An alternative procedure to the above is to take the affinity matrix which has retained the strongly bound pAb and extract the DNA, for example by boiling in SDS solution. Extracted DNA can then be used to directly transform E.coli host cells or alternatively the antibody

25   encoding sequences can be amplified, for example using PCR with suitable primers such as those disclosed herein, and then inserted into a vector for expression as a soluble antibody for further study or a pAb for further rounds of selection.

30   Another preferred method for selection according to affinity would be by binding to an affinity matrix containing low amounts of ligand.

         If one wishes to select from a population of phages displaying a protein molecule with a high affinity for

35   its ligand, a preferred strategy is to bind a population of phage to an affinity matrix which contains a low amount of ligand. There is competition between phage, displaying high affinity and low affinity proteins, for

binding to the ligand on the matrix. Phage displaying high affinity protein is preferentially bound and low affinity protein is washed away. The high affinity protein is then recovered by elution with the ligand or

5   by other procedures which elute the phage from the affinity matrix (Example 35 of WO92/01047 demonstrates this procedure).

In summary then, for recovery of the packaged DNA from the affinity step, the package can be simply eluted,

10 it can be eluted in the presence of a homologous sbp member which competes with said package for binding to a complementary sbp member; it could be removed by boiling, it could be removed by proteolytic cleavage of the protein; and other methods will be apparent to those

15 skilled in the art eg. destroying the link between the substrate and complementary sbp member to release said packaged DNA and sbp member. At any rate, the objective is to obtain the DNA from the package so that it can be used directly or indirectly, to express the sbp member

20 encoded thereby.

The efficiency of this selection procedure for pAbs and the ability to create very large libraries means that the immunisation techniques developed to increase the proportion of screened cells producing antibodies of

25 interest will not be an absolute requirement. The technique allows the rapid isolation of binding specificities eg antigen-binding specificities, including those that would be difficult or even unobtainable by conventional techniques, for example, catalytic or anti-

30 idiotypic antibodies. Removal of the animal altogether is now possible, once a complete library of the immune repertoire has been constructed.

Applications of antibodies to self antigens

35

Human antibodies to cell surface components The isolation of such antibody specificities would be particularly useful for preparing agents which mediate

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cell killing for instance of cancer cells, for example
using the natural effector function of antibodies.
Anti-self antibodies may also be valuable in the
preparation of diagnostic in vivo imaging reagents, for
5   instance using radioisotopes.
        Antibodies directed against cell surface components
of specific T-cell subsets could be used therapeutically
(D.Wraith et al Cell 57 709-715,1989; L.Steinman and
R.Mantegazza FASEB J. 4 2726-2731,1990), for instance to
10   prevent T cell action causing rheumatoid arthritis.

Human antibodies modifying the function of self molecules
        Antibodies can be isolated which modify the action
of self molecules such as hormones, growth factors and
15   receptors through their binding to a specific epitope on
the molecule. Multifunctional proteins may have both
desirable and undesirable characteristics, particularly
.if they are used therapeutically. For instance, the
lymphokine TNF (tumour necrosis factor) binds to, at
20   least two different classes of cell receptors- one
commonly found on vascular endothelial cells, the other
commonly found on tumour cells. A mouse antibody to TNF
has been made which prevents TNF from binding to
endothelial cell receptors while still allowing it to
25   bind to tumour cells thus allowing attack on the tumours
without toxic side effects mediated through endothelial
cells (Patent application PCT/AU90/00337). For
therapeutic use of antibody modifiers of hormone or
growth factor molecules, it would be preferable to have a
30   human antibody specificity isolated directly through
selection from a phage library.

Human anti-idiotypes
        Anti-idiotype antibodies (antibodies directed
35   against the antigen combining sites formed by the
variable domains of human antibodies) are conventionally
made by isolating an antibody against an antigen and then
using this isolated antibody as an immunogen to raise

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antibodies directed against it. If the original antibody is directed against a hormone or growth factor, the relationship between antigen and antibody combining sites means that the anti-idiotype may mimic in some aspects

5   the hormone or growth factor and bind to the receptor for these molecules. However, the fraction of anti-idiotype antibodies able to mimic the binding of the hormone to the receptor would be expected to be small. Furthermore, the deletion of antiself lymphocytes would

10 mean that using the conventional route to antiidiotypes would be difficult for the isolation of human anti-idiotype antibodies mimicking molecules binding human receptors. In this application we show that antibodies directed against the antigen combining sites

15 formed by the variable domains of human antibodies may be directly isolated from phage antibody display libraries, as shown in examples 1 and 4, and it should also be possible to identify the anti-idiotypic antibodies mimicking the binding of the hormone directly by

20 screening for binding to the receptor.
         Anti-idiotypes may also be useful for the treatment of autoimmune disease. They could be used to bind to circulating autoantibodies. However, it may be preferable to attack directly antibody producing cells,

25 for instance using a bispecific antibody directed against a cell surface marker as well as an anti-idiotype specificity. Alternatively, plasmaphoresis could be used to remove circulating antibody and the cells treated directly.

30

Human antibodies against receptors
         Human antibodies that bind to receptors, blocking or antagonising ligand function could be selected directly from a phage library displaying antibodies derived from

35 an unimmunised donor.

Human antibodies to prevent transplant rejection
         Antibodies directed against the major

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histocompatibility complex proteins could be used to
treat patients following transplants, or organs prior to
transplantation, in order to prevent rejection.
Antibodies directed against several lymphocyte cell
5   surface markers have been used for the prevention of
rejection in transplants e.g. CD45, CD3, CD4, CD8 and
interleukin-2 receptor. Example 3 shows that human
antibodies against CD4 can be directly isolated from
phage display libraries.
10

Human antibodies against cytokines
        Human antibodies against cytokines would be valuable
for treatment of human disease, for example of septic
shock with anti-TNF and anti-interleukin 1 antibodies.
15   Examples 1 and 6 show that human antibodies against TNF
can be isolated directly from phage antibody libraries
derived from unimmunised humans or the synthetic
recombination of V,D and J fragments. In many cases
these cytokine molecules are strongly conserved between
20   species, for instance transforming growth factor-β
(TGF-β), and it has proved difficult to isolate
antibodies directed against the human molecule even in
mice. The isolation of human anti-self antibodies as
described in this invention provides a method of
25   obtaining human antibodies with such a specificity.

Human antibodies for diagnosis and treatment of cardiac
disorders
        Human antibodies against clot components e.g.
30   fibrin, would be useful for imaging clots when labelled
with radioactivity or for dissolving clots, if for
example linked to a clot dissolving enzyme such as
urokinase.

35   Antibodies triggering receptor function
        Antibodies may be selected that bind to a cell
receptor and trigger a biological response in the cell.
This is described in more detail below and in Example 8

describes the isolation of such antibodies.

By cycles of growth and selection, those rgdps binding to the cell receptors are isolated. Some of these rgdps encode binding specificities with the potential (alone or in combination with other binding specificities) to trigger the receptors. These binding specificities are tested alone, or in combination, for triggering the cell receptors.

There are a variety of cell receptors in which the binding of a ligand, for example hormone, growth factor, or peptide triggers a biological event, for example the activation of tyrosine kinase activity, or the opening of an ion channel. The rdgps could be selected for binding to cell receptor (or a related receptor with conserved portions of surface such as from another species), for example by using cells displaying the cell receptor, or using soluble receptor immobilised on solid phase, or using domains or peptide epitopes of the receptor. Ideally the receptor would be provided in a crosslinked form (as required for its triggering).

Triggering of receptors at the cell surface often seems to involve the relative movement of proteins or subunits. For example, in the neurotransmitter-gated receptors, the five subunits that are arranged symmetrically in the membrane place, delineate an ion pathway down the centre. Binding of the neurotransmitter is thought to alter the size of the central ion channel by causing small rearrangements between the subunits in an allosteric transition. For tyrosine kinase receptors, the ligand appears to drive receptor oligomerisation. Thus antibodies with binding specificities directed against a receptor may have the potential to promote an allosteric change or to promote oligomerisation. The oligomerisation of the receptors may also be promoted by using bivalent or bispecific antibodies.

The soluble antibodies or antibody fragments may be monovalent fragments, for example, single chain Fv fragments or Fab fragments, or bivalent fragments, for

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example, Fab_2 or complete antibody fragments. The bivalency could also be promoted in other ways, for example (1) by encoding a tag, such as a peptide or protein (for example, the subunit of a dimeric protein)

5   that self associates, at the N or C-terminus of the monomeric fragment, (2) using a bivalent antibody that binds to the monovalent fragment, for example, to a common C-terminal tag, or to an antibody constant domain (3) chemical cross-linking.

10   Bispecific antibody or bispecific fragments could also be made as for the bivalent fragments. (For expression of the bispecific antibody or fragment in the same cell, the genes encoding both specificities would need to be introduced together). The different antibody

15   "arms" could be directed against the same receptor, for example to different epitopes, or to two different receptors (to trigger hybrid receptors).

The direct isolation of anti-self antibodies from phage libraries as described in this invention is

20   important to allow a large number of antibodies to be surveyed for these triggering receptors.

It is appropriate to distinguish the making of antibodies to trigger receptors as is described here and provided as an aspect of the present invention from the

25   "anti-idiotypic route" in which specific antibodies raised in an animal, including man, by vaccinating the said animal with a specific antigen are themselves used to vaccinate another animal, new antibodies termed anti- idiotypic antibodies (Anti-Ids) being produced able to

30   recognise and bind to the first set of antibodies. Some species of these Anti-Ids are able to mimic the specific biological properties of the original antigen. If for example, the antigen were a peptide hormone or a cell receptor, the Anti-Id to the hormone or cell receptor

35   antigen is able to elicit a response of the cell (See Gaulton, G.N. and Greane, M.I., 1986. Idiotypic mimicry of biological receptors. Ann. Rev. Immunol. 4,253-280; Sege, K. and Peterson, P.A., 1978. Use of anti-

idiotypic antibodies as cell surface receptor probes. Proc. Natl. Acad. Sci. Usa. 75, 2443-2447 for examples).

The essence of current teaching of Anti-Ids as mimics of antigens is that they are produced as a result of constructing antibodies to antibodies of the original antigen. There is however, some controversy over whether such anti-idiotypes accurately mimic the original antigen (S.J. Davis et al Nature 358 76-79, 1992).

There is therefore a clear distinction between antibodies prepared by an anti-idiotypic route that mimic antigens such as growth factors or hormones, and antibodies that are made directly to the receptors to trigger the receptors. The antibodies derived by an anti-idiotypic route require the antigen (hormone, growth factor) and will bind to the same epitope on the receptor as the hormone, while the antibodies derived by binding to the receptors need not bind to the same epitope to trigger the receptor. Indeed such antibodies need not mimic a known hormone or growth factor, as their specificity, or binding to receptor (characterised as epitope, on-rate or off-rate) or blood clearance is likely to differ. The process for making the antibodies is also quite different. Anti-idiotypic antibodies are made classically by immunisation of animals, although they can be isolated directly from phage display libraries as described above. Antibodies directed against self receptors are made by selection from V-gene libraries (as described above).

As well as the advantages over the anti-idiotypic route, the antibodies derived directly by receptor binding may even have advantages over the natural hormone or growth factor. Thus receptors that are defective for binding of the natural hormone or growth factor (for example in a genetic disease), may be triggered by an antibody binding at a different epitope.

As therapeutic agents the various isotypes of antibodies or fragments of antibodies carrying the variable regions responsible for the specificity of the

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molecule have a number of properties having advantages over the bioactive moiety they mimic. For example, unlike the natural hormones their half-life in circulation can be modified readily. Depending on the

5   antibody isotype or fragment chosen, they have half-lives in circulation in a patient ranging from minutes to several weeks. If long term usage or short term clearance is required this can easily be accommodated by choosing the appropriate antibody isotype without need to

10   use slow release devices as implants, or continuous intravenous infusion, etc.

Furthermore, many hormones or tissue growth factors or antigens in general are functionally complex with different epitopes of each of the molecules having

15   various specific functions. Clones of antibody mimics are monofunctional in this respect so could be used to produce one specific biological effect of a hormone without a second effect which latter effect may be disadvantageous to the patient. Thus the lymphokine TNF

20   (tumour necrosis factor) binds to two different classes of cell receptors - one common on vascular endothelial cells, the other common on tumour cells. If the TNF is modified so that it cannot bind to the endothelial cell receptors but can still bind to tumour cell receptors,

25   the tumours are attacked without at the same time inducing the very toxic side effects mediated through the vascular receptors. (This is described in Australian Patent Application PCT/AU90/00337). An antibody mimic able to recognise the tumour cell receptor would be

30   expected to be very specific and kill tumour cells without inducing toxic side effects mediated through the vascular endothelium since it would have no resemblance to the TNF epitope which binds to receptors on the latter.

35

TERMINOLOGY

Much of the terminology discussed in this section has been mentioned in the text where appropriate.

5

Self

A self antigen is an antigen or epitope which is capable of binding to an antigen binding site formed by antibody variable domain(s) and which is conserved

10   between members of a species of animal and native to the body.

The immune system tries to avoid making antibodies to self antigens. It has been suggested that (i) sequences of germ line V gene segments have been evolved

15   under pressure to be directed towards foreign, e.g. pathogen, antigens and epitopes, and away from being able to provide antibodies which will bind self antigens, and (ii) that, in addition to this, immune tolerance causes those combinations of gene segments encoding anti-self

20   antibody which do arise, to be deleted or anergised. Consequently, there are not normally circulating antibodies against these antigens except in disease states, eg autoimmune diseases. A self antigen may be one which does not vary between individuals of a species.

25   A self antigen may be one for which there is normal allelic variation throughout a population. Immunisation of one individual in a species with a self antigen would not normally be expected to result in generation, or detection, of antibodies to the antigen, except perhaps

30   when tolerance is deliberately broken. Antibodies to a self-antigen may only be present in an individual who is suffering from autoimmune disease. On the other hand, there are some self antigens to which circulating antibodies can be found in a sub-population of normal

35   individuals of a species.

A self antigen may be an antigen recognised by B-cell surface antibodies but not by antibodies which can be found circulating. It might not be possible to detect

or obtain circulating antibodies to a self antigen except perhaps when the individual is suffering from an autoimmune disease or syndrome.

5   An anti-self antibody or antibody fragment is an antibody or fragment thereof which has binding specificity for a self antigen. It may recognise an epitope which is found only on a self antigen, or it may be cross-reactive with an antigen which individuals of the species will recognise as foreign. The present

10   invention is particularly well suited to the production and isolation of antibody fragments which bind only a self antigen.

Specific Binding Pair

15   This describes a pair of molecules (each being a member of a specific binding pair) which are naturally derived or synthetically produced. One of the pair of molecules, has an area on its surface, or a cavity which specifically binds to, and is therefore defined as

20   complementary with a particular spatial and polar organisation of the other molecule, so that the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor,

25   receptor-ligand, enzyme-substrate, lgG-protein A.

Multimeric Member

This describes a first polypeptide which will associate with at least a second polypeptide, when the

30   polypeptides are expressed in free form and/or expressed on the surface of a substrate. The substrate may be provided by a bacteriophage. Where there are two associated polypeptides, the associated polypeptide complex is a dimer, where there are three, a trimer etc.

35   The dimer, trimer, multimer etc or the multimeric member may comprise a member of a specific binding pair.

Example multimeric members are heavy domains based on an immunoglobulin molecule, light domains based on an

immunoglobulin molecule, T-cell receptor subunits.

Replicable Genetic Display Package (Rgdp)

5   This describes a biological particle which has genetic information providing the particle with the ability to replicate. The particle can display on its surface at least part of a polypeptide. The polypeptide can be encoded by genetic information native to the particle and/or artificially placed into the particle or

10   an ancestor of it. The displayed polypeptide may be any member of a specific binding pair eg. heavy or light chain domains based on an immunoglobulin molecule, an enzyme or a receptor etc.

       The particle may be a virus eg. a bacteriophage such

15   as fd or M13.

Package

       This describes a replicable genetic display package in which the particle is displaying a member of a specific binding pair at its surface. The package may be

20   a bacteriophage which displays an antigen binding domain at its surface. This type of package has been called a phage antibody (pAb).

Antibody

25   This describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any protein having a binding domain which is, or is homologous to, an immunoglobulin binding domain. These proteins can be derived from natural sources, or

30   partly or wholly synthetically produced.

       Example antibodies are the immunoglobulin isotypes and the Fab, $F(ab^1)_2$, scFv, Fv, dAb, Fd fragments.

Immunoglobulin Superfamily

35   This describes a family of polypeptides, the members of which have at least one domain with a structure related to that of the variable or constant domain of

immunoglobulin molecules. The domain contains two β-
sheets and usually a conserved disulphide bond (see A.F.
Williams and A.N. Barclay 1988 Ann. Rev Immunol. 6, 381-
405).

5   Example members of an immunoglobulin superfamily are
CD4, platelet derived growth factor receptor (PDGFR),
intercellular adhesion molecule. (ICAM). Except where
the context otherwise dictates, reference to
immunoglobulins and immunoglobulin homologs in this
10   application includes members of the immunoglobulin
superfamily and homologs thereof.

Homologs
       This term indicates polypeptides having the same or
15   conserved residues at a corresponding position in their
primary, secondary or tertiary structure. The term also
extends to two or more nucleotide sequences encoding the
homologous polypeptides.
       Example homologous peptides are the immunoglobulin
20   isotypes and the TIM barrel enzymes.

Functional
       In relation to a sbp member displayed on the surface
of a rgdp, means that the sbp member is presented in a
25   folded form in which its specific binding domain for its
complementary sbp member is the same or closely analogous
to its native configuration, whereby it exhibits similar
specificity with respect to the complementary sbp member.

30

Genetically diverse population
       In connection with sbp members or polypeptide
components thereof, this is referring not only to
diversity that can exist in the natural population of
35   cells or organisms, but also diversity that can be
created by artificial mutation in vitro or in vivo.
       Mutation in vitro may for example, involve random
mutagenesis using oligonucleotides having random

mutations of the sequence desired to be varied. _In vivo_
mutagenesis may for example, use mutator strains of host
microorganisms to harbour the DNA (see Example 38 of WO
92/01047). The words "unique population" may be used to
denote a plurality of eg polypeptide chains which are not
genetically diverse, ie they are all the same. A
restricted population is one which is diverse but less so
thatn the full repertoire of an animal or a library,
synthetic or otherwise. The diversity may have been
reduced by prior selection, eg using antigen binding
specificity.

<u>Domain</u>

A domain is a part of a protein that is folded
within itself and independently of other parts of the
same protein and independently of a complementary binding
member. A folded unit is a specific combination of a α-
helix and/or β-sheet structure. Domains and folded units
contain structures that bring together amino acids that
are not adjacent in the primary structure.

<u>Free Form</u>

This describes the state of a polypeptide which is
not displayed by a replicable genetic display package.

<u>Conditionally Defective</u>

This describes a gene which expresses a defective
polypeptide under one set of conditions, but expresses a
different but related non-defective polypeptide under
another set of conditions. An example, is a gene
containing an amber mutation expressed in non-suppressing
or suppressing hosts respectively.

Alternatively, a gene may express a protein which is
defective under one set of conditions, but not under
another set. An example is a gene with a temperature
sensitive mutation.

<u>Suppressible Translational Stop Codon</u>

This describes a codon which allows the translation

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of nucleotide sequences downstream of the codon under one set of conditions, but under another set of conditions translation ends at the codon. Example of suppressible translational stop codons are the amber, ochre and opal

5   codons.

Mutator Strain

This is a host cell which has a genetic defect which causes DNA replicated within it to be mutated with

10 respect to its parent DNA. Example mutator strains are NR9046mutD5 and NR9046 mut T1 (see Example 38 of WO92/01047).

Helper Phage

This is a phage which is used to infect cells

15 containing a defective phage genome and which functions to complement the defect. The defective phage genome can be a phagemid or a phage with some function encoding gene sequences removed. Examples of helper phages are M13K07, M13K07 gene III no. 3; and phage displaying or encoding

20 a binding molecule fused to a capsid protein.

Vector

This is a DNA molecule, capable of replication in a host organism, into which a gene is inserted to construct

25 a recombinant DNA molecule.

Phage Vector

This is a vector derived by modification of a phage genome, containing an origin of replication for a

30 bacteriophage, but not one for a plasmid.

Phagemid Vector

This is a vector derived by modification of a plasmid genome, containing an origin of replication for a

35 bacteriophage as well as the plasmid origin of replication.

Secreted

This describes a rgdp or molecule that associates
with the member of a sbp displayed on the rgdp, in which
the sbp member and/or the molecule, have been folded and
the package assembled externally to the cellular cytosol.

Repertoire of Rearranged Immunoglobulin Genes

A collection of naturally occurring nucleotides eg
DNA sequences which encoded expressed immunoglobulin
genes in an animal. The sequences are generated by the
in vivo rearrangement of eg V, D and J segments for H
chains and eg the V and J segments for L chains.
Alternatively the sequences may be generated from a cell
line immunised in vitro and in which the rearrangement in
response to immunisation occurs intracellularly.

Library

A collection of nucleotides eg DNA sequences within
clones; or a genetically diverse collection of
polypeptides, or specific binding pair members, or
polypeptides or sbp members which are displayed on rgdps
capable of being selected or screened to provide an
individual polypeptide or sbp member or a mixed
population of polypeptides or sbp members.

Repertoire of Artificially Rearranged Immunoglobulin
Genes

A collection of nucleotides eg DNA sequences derived
wholly or partly from a source other than the rearranged
immunoglobulin sequences from an animal. This may
include for example, DNA sequences encoding VH domains by
combining unrearranged V segments with D and J segments
and DNA sequences encoding VL domains by combining V and
J segments.

Part or all of the DNA sequences may be derived by
oligonucleotide synthesis.

Secretory Leader Peptide

This is a sequence of amino acids joined to the N-

terminal end of a polypeptide and which directs movement
of the polypeptide out of the cytosol.

Eluant

5   This is a solution used to breakdown the linkage
between two molecules. The linkage can be a non-
covalent or covalent bond(s). The two molecules can be
members of a sbp.

10 Derivative

This is a polypeptide which derived from another
polypeptide which is encoded by the DNA within a
selected rdgp. The derivative polypeptide may differ
from the encoded polypeptide by the addition, deletion,

15 substitution or insertion of amino acids, or by the
linkage of other molecules to the encoded polypeptide.
These changes may be made at the nucleotide or protein
level. For example the encoded polypeptide may be a Fab
fragment which is then linked to an Fc tail from another

20 source. Alternatively markers such as enzymes,
fluoresceins etc may be linked to e.g. Fab, scFv
fragments.

BRIEF DESCRIPTION OF THE FIGURES

25

Figure 1 shows an analysis by ELISA of the
specificities of soluble single-chain Fvs (scFvs)
isolated from the unimmunised library by selection on
bovine thyroglobulin (upper panel), human TNFα (central

30 panel), or the human mAb Fog-1 (gamma-1, kappa).
Binding was determined by ELISA to a panel of proteins,
as follows: 1 - plastic; 2 - hen egg trypsin inhibitor;
3 - chymotrypsinogen A; 4 - hen egg ovalbumin;
5 - keyhole limpet haemocyanin; 6 - bovine

35 thyroglobulin; 7 - human TNFα; 8 - turkey egg-white
lysozyme; 9 - horse heart cytochrome c; 10 - bovine
serum albumin; 11 - mAb Fog-1.
Figure 2 shows an analysis by ELISA of the

40

specificities of soluble scFvs isolated from the
unimmunised library by selection on human
carcinoembryonic antigen (CEA) (upper panel), the MUC1
peptide (Price et al., 1990, supra) (central panel), or
human CD4 (lower panel). Binding was determined by
ELISA to a panel of proteins, as follows: 1 - hen egg
trypsin inhibitor; 2 - chymotrypsinogen A; 3 - hen egg
ovalbumin; 4 - keyhole limpet haemocyanin; 5 - CEA;
6 - urine extract containing human polymorphic
epithelial mucin (PEM); 7 - bovine thyroglobulin;
8 - hen egg-white lysozyme; 9 - bovine serum albumin;
10 - chicken gamma globulin coupled to 4-hydroxy-3-
nitrophenyl acetic acid; 11 - human recombinant soluble
CD4.

Figure 3 shows an ELISA to assay the binding of three
scFvs, isolated by selection on a human monoclonal
antibody Fog-1 (IgG1, kappa), to a panel of human
antibodies of varying isotype, as follows: 1 - Fog-1;
2 - the Fv fragment of Hulys11; 3 - Hulys11 antibody
(IgG1, kappa); 4 - RegA (IgG1, kappa); FogC (IgG3,
kappa); 6 - Pag1 (IgG1, lambda); 7 IgG2,lambda antibody
purified from myeloma plasma (Sigma); 8 - Oak3 (IgG3,
lambda); 9 - IgG4, lambda purified from myeloma plasma
(Sigma); 10 Fom1 (IgM, lambda); 11 - FomA (IgM, lambda).

Figure 4 illustrates the assembly of VH genes in the
creation of a synthetic library.

Figure 5 shows schematically selection techniques for
pAbs: 2(i) shows a binding/elution system; 2(ii) shows a
competition system (p = pAb; ag = antigen to which
binding by pAb is required; c = competitor population
e.g. antibody, pAb, ligand; s = substrate (e.g. plastic
beads etc); d = detection system).

The present invention is illustrated by the
following examples. Oligonucleotide primers and probes
mentioned in the text are listed in Table IV. Tables I
to IV are found after Example 8.

Example 1 shows the isolation of antibodies directed

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against human tumour necrosis factor-α and a human
monoclonal antibody from a phage library of single chain
Fv fragments derived from an unimmunized human.

5 Example 2 shows the isolation of antibodies binding to
human thyroglobulin from a phage library of single chain
Fv fragments derived from an unimmunized human.

Example 3 shows the isolation of antibody fragments
10 directed against the self antigens MUC1 mucin,
carcinoembryonic antigen (CEA) and recombinant soluble
CD4 (rsCD4) from a phage display library of single chain
Fv fragments derived from an unimmunized human.

15 Example 4 shows the further characterization of selected
anti-self antibody fragments by DNA sequencing and
affinity determinations.

Example 5 shows the creation of a synthetic human
20 library using germ line VH segments.

Example 6 shows the isolation of an antibody fragment
binding to human tumour necrosis factor-α from a human
germ line synthetic library.

25
Example 7 shows the creation of a synthetic human
library using human germ line VH segments containing VH
CDR3 sequences of different lengths and isolation of
single chain Fv fragments binding to human thyroglobulin
30 and a human monoclonal antibody.

Example 8 shows the isolation of human antibodies
directed against human interleukin-1 receptor molecules
which trigger receptor function.

35
Example 1
Isolation of antibody fragments directed against self
antigens from a library of scFvs made from unimmunized

blood donors

Naturally occuring V-genes isolated from human PBLs can be constructed into a large library of antibody fragments which contain reactivities against antigens to which the donor has not been exposed (WO92/01047 example 5 42). We have realised that these libraries may also contain reactivities against self antigens, arising either from self-reactive B-cells which have not been deleted or as non-naturally occuring fragments resulting 10 from VH and VL chain recombination. To test this, we panned a large human scFv library displayed on the surface of a phagemid against human TNF-a and a human IgG/k immunoglobulin.

15 **Methods**
Rescue of the library:

The library of scFvs was constructed from the RNA of human PBLs and has been previously described (WO92/01047 example 42). To rescue phage displaying 20 antibody fragments, approximately 10^9 E. coli harbouring the phagemid were used to inoculate 50 ml of 2 x TY containing 1% glucose and 100 mg/ml of ampicillin (2 x TY- AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture was used to innoculate 25 50 ml of 2 x TY- AMP-GLU, 2 x 10^8 TU of delta gene 3 helper (M13 D gene III see WO92/01047) were added and the culture incubated at 37°C for 45 minutes without shaking and then at 37°C for 45 minutes with shaking. The culture was centrifuged at 4000 r.p.m. for 10 min. 30 and the pellet resuspended in 2 litres of of 2 x TY containing 100 mg/ml ampicillin and 50 mg/ml kanamycin and grown overnight. Phage were prepared as previously described (WO92/01047 example 42). M13 D gene III was prepared as follows:

35 M13 D gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 D gene III particles are made by growing

43

the helper phage in cells harbouring a pUC19 derivative
supplying the wild type gIII protein during phage
morphogenesis. The culture was incubated for 1 hour at
37°C without shaking and then for a further hour at 37°C

5   with shaking. Cells were spun down (IEC-Centra 8, 4000
revs/min for 10 min), resuspended in 300 ml 2 x TY broth
containing 100mg ampicillin/ml and 25mg kanamycin/ml (2
x TY-AMP-KAN) and grown overnight, shaking at 37°C.
Phage particles were purified and concentrated from the

10 culture medium by two PEG-precipitations (Sambrook et
al., 1990), resuspended in 2 ml PBS and passed through a
0.45mm filter (Minisart NML; Sartorius) to give a final
concentration of approximately 10^{13} transducing units/ml
(ampicillin-resistant clones).

15

Panning of the library:
        Immunotubes (Nunc) were coated overnight in PBS
with 4 ml of either 100 mg/ml or 10 mg/ml of recombinant
human TNF-a in PBS or 4 ml of 10 mg/ml of Fog-1, a human

20 IgG/k immunoglobulin which recognizes the human red
blood cell Rh (D) antigen. Tubes were blocked with 2%
Marvel-PBS for 2 hours at 37° C and then washed 3 times
in PBS. Approximately 1013 TU of phage was applied to
the tube and incubated for 30 minutes at room

25 temperature tumbling on an over and under turntable and
then left to stand for another 1.5 hours. Tubes were
washed 10 times with PBS 0.1% Tween-20 and 10 times with
PBS. Phage were eluted by adding 1 ml of 100 mM
triethylamine and rotating 15 minutes on an under and

30 over turntable after which the solution was immediately
neutralized with 0.5 ml of 1,0 M Tris-HCl, pH 7.4.
Phage were then used to infect 10 ml of mid-log E. coli
TG1 by incubating eluted phage with bacteria for 30
minutes at 37° C. The E. coli were then plated on TYE

35 plates containing 1% glucose and 100 mg/ml ampicillin.
The resulting bacterial library was then rescued with
delta gene 3 helper phage as described above to prepare
phage for a subsequent round of selection. This process

44

was then repeated for a total of 4 rounds of affinity
purification with tube-washing increased to 20 times
with PBS, 0.1% Tween-20 and 20 times with PBS for rounds
3 and 4.

5

Characterization of binders:

Eluted phage from the 3rd and 4th rounds of
selection were used to infect E. coli HB 2151 and
soluble scFv was produced (Marks, et al., 1991) from

10 single colonies for assay. In the case of TNF, phage
was also rescued from single colonies. ELISAs were
performed as previously described with microtitre plates
coated with either 10 µg/ml human TNF-a in 50 mM
bicarbonate pH 9.6 or 10 µg/ml Fog-1 in PBS. Clones

15 positive in ELISA were further characterized by PCR
fingerprinting (WO92/01047 example 20) and then by
sequencing.

Results

20 TNF: Soluble scFv from 1536 colonies and phage from
1152 colonies were screened by ELISA. The results are
shown in figure 1, the key for which is given in the
brief description of the figures (supra). Positive
clones for binding to TNF-a were further characterized

25 by PCR fingerprinting and sequencing. In this manner,
15 different binders were identified. Four of these
have been sequenced.

Fog-1: Soluble scFv from 96 clones was screened by

30 ELISA and positive clones were further characterized by
PCR fingerprinting and sequencing. In this manner, four
different binders were identifed and sequenced.

Example 2
35 Isolation of antibody fragments specificities directed
against human thyroglobulin from a library of scFv
fragments using display on bacteriophage fd

Example 44 of WO92/01047 describes the selection of

45

antibody scFv fragments directed against bovine
thyroglobulin from a library of scFv fragments. These
were derived from unimmunised humans, expressed on the
surface of phage fd, isolated by panning against bovine
5   thyroglobulin. The results demonstrated that it is
possible to isolate from a library derived from an
unimmunised individual antibody fragments which will
bind an antigen to which that individual has never been
exposed.

10   Sixteen clones found by this panning to be specific
for bovine thyroglobulin have now been analysed for
binding to human thyroglobulin in an ELISA assay (as
described in example 44 of WO92/01047). Nine of these
clones also bound strongly to human thyroglobulin with
15   absorbance signals of between 1.0 and 1.6 12 minutes
after addition of substrate. No cross-reactivity
(signal less than 0.05 after 90 min) was found with a
panel of unrelated antigens- hen egg lysozyme, BSA,
ovalbumin, chymotrypsinogen, cytochrome c, keyhole
20   limpet hemocyanin, insulin, cardiolipin and DNA.

Thus, antibodies with specificity for epitopes on
the human self antigen thyroglobulin can be isolated
from libraries prepared from unimmunised humans.

Two clones binding to both human and bovine
25   thyroglobulin, α-Thy23 and α-Thy29, and two clones
binding to bovine thyroglobulin only, α-Thy32 and α-
Thy33, were sequenced.

Example 3
30   Isolation of antibody fragments directed against the
human self-antigens MUC1 mucin, carcinoembryonic antigen
(CEA) and recombinant soluble CD4 (rsCD4) from a phage
display library of human single chain Fv fragments
The phage display library of single chain Fv
35   fragments derived from unimmunized human donors used in
Example 1 was used in selection to isolate antibody
fragments directed against the self antigens MUC1 mucin,
carcinoembryonic antigen (CEA) and recombinant soluble

CD4 (rsCD4).

Rescue of the library

The library was rescued as in example 1 except that
the standard helper phage M13K07 (5 x 10^{10} pfu) was used
to rescue the library rather than delta gene 3 helper
phage (M13 D gene III).

Selection of phage specific for MUC1 mucin and
carcinoembryonic antigen (CEA)

The phage were panned for binding using immuno
tubes (Nunc; Maxisorp) coated with antigen essentially
as (Marks et al., 1991), or were selected on a column of
antigen (J.McCafferty et al., Nature 348 552-554, 1990).
The following antigens were used: human recombinant
soluble CD4 (rsCD4) (expressed in baculovirus by
American Biotechnologies Inc. and supplied by the MRC
AIDS Reagent Project [ADP608]); human carcinoembryonic
antigen (CEA); and a 20 amino acid peptide (M.R.Price et
al., Molec. Immunol. 27 795-802, 1990), which
corresponds to a repeated motif in human MUC1 mucin
(tumour-associated polymorphic epithelial mucin or PEM)
(S. Gendler et al., J. Biol. Chem. 263 12820-12823,
1988; J.R.Gum et al., Biochem. Biophys. Res. Commun. 171
407-415, 1990).

CEA (20mg/ml) and rsCD4 (10mg/ml) were coated on
immunotubes overnight at room temperature in phosphate
buffered saline. For the first two rounds of selection
tubes were washed 10 times with PBS, 0.1% (v/v) Tween 20
and 10 times with PBS. For subsequent rounds of
selection tubes were washed 20 times with PBS, 0.1%
(v/v) Tween 20 and 20 times with PBS. Phage were eluted
with 100 mM triethylamine as (Marks et al., 1991).
Eluted phage (usually 10^6 to 10^7 transducing units) were
used to infect E. coli TG1 cells. Approx. 10^9 infected
bacteria were used as an inoculum for the next rescue.
The library was subjected to 3 to 5 rounds of rescue and
selection for each antigen.

47

For selection of phage binding to the MUC1 peptide, the peptide was coupled chemically to Sepharose 4B (provided by M.R. Price). A 1ml column was prepared, and phage was selected as described by McCafferty et

5   al., 1990 (supra). Briefly, the Sepharose-MUC1 column was washed with PBS containing 2% skimmed milk powder (MPBS) and the phage loaded in 1ml of the same buffer. After washing the column successively with 10ml volumes of MPBS, PBS pH7.2, 50mM Tris-HCl/500mM NaCl pH8.0, and

10 50mM Tris-HCl/500mM NaCl pH9.0, phage was eluted with 5ml 100mM triethylamine and neutralised with 0.5M sodium phosphate buffer pH 6.8. Five rounds of selection were carried out.

15 Screening and sequencing of clones

Single ampicillin resistant colonies from infection of E. coli TG1 with eluted phage, were screened either for binding of phage (Clackson et al., 1991) or soluble scFv fragments (Marks et al., 1991). Since the gene

20 encoding the antibody fragment is linked to that encoding the phage coat protein by an amber codon, soluble fragments can be secreted from a non-suppressor strain of bacteria infected by the phage (Hoogenboom et al., 1991). The binding to antigen of soluble scFvs in

25 bacterial supernatant was detected with the mouse mAb 9E10 (1 µg/ml), which recognises the C-terminal peptide tag (Munro and Pelham, Cell 46, 291-300, 1986), and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described (Ward et al., 1989). Plates were coated with

30 the antigens Fog1, TNFa, bovine thyroglobulin and rsCD4 as described for immuno tubes above, and with CEA at 5mg/ml. A urine extract containing human polymorphic epithelial mucin (PEM) was used at a protein concentration of approximately 10mg/ml.

35 The specificity of the isolated clones was checked by ELISA of the soluble scFv fragments using plates coated with various proteins. Plates were coated with the antigens Fog-1, TNFa, bovine thyroglobulin, rsCD4,

48

CEA and PEM as described above. Other proteins were coated overnight at room temperature at a concentration of 1 mg/ml in PBS (cytochrome c [Sigma]) or in 50 mM NaHCO3, pH 9.6 (bovine serum albumin, turkey egg-white

5 lysozyme, hen-egg-white lysozyme, hen ovalbumin, keyhole limpet haemocyanin [CalBiochem], chymotrypsinogen A, chicken egg-white trypsin inhibitor [Sigma], chicken gamma globulin coupled to 4-hydroxy-3-nitrophenyl acetic acid.   Clones found to give a positive ELISA signal

10 were screened by PCR and 'fingerprinted' with the restriction enzyme BstNI as in (Marks et al., 1991, supra) to identify different clones. Examples of clones with different restriction patterns were selected and the heavy and light chains sequenced using a Sequenase

15 kit (USB) or using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an Applied Biosystems 373A DNA sequencer.

Sequenced clones were further analysed using the program MacVector 3.5 (IBI Kodak, New Haven, CT). The

20 VH genes were compared to the 83 germline gene segments present in the VH directory compiled by Tomlinson et al. (J.Mol.Biol. 227 776-798, 1992). VL genes were compared with 34 published kappa germline gene segments and 13 published lambda gene segments. Regions of the V-genes

25 encoded by PCR primers were not included in the analysis.

The selected human antibody fragments show high specificity against self-antigens

30 After two to five rounds of selection, E.coli cells were infected with eluted phage and antibody fragments produced by individual clones were screened for binding by ELISA. Phage selected with the 20 amino acid MUC1 peptide (Price et al., 1990,supra), which corresponds to

35 a repeated motif in human MUC1 mucin (tumour-associated polymorphic epithelial mucin or PEM) (Gendler et al., 1988, supra; Gum et al., 1990, supra), were screened for binding to human PEM and hence bind to both peptide and

the protein. The V-genes of clones with binding
activities were sequenced, and one clone identified for
each antigen of CEA, PEM and rsCD4 (Table I). The
appearance of only low numbers of clones binding to CEA,

5   PEM and human recombinant soluble CD4 (rsCD4), even
after several rounds of selection, may reflect the use
of VCS-M13 (stratagene) as helper phage (instead of
M13DgIII helper used for the other antigens).
Populations of phage(mid) particles produced by rescue

10   with M13DgIII (which cannot produce pIII) have higher
average avidities than those produced by rescue with
VCS-M13 (where the wild-type pIII encoded by the helper
phage can compete with scFv-pIII fusions).

        The scFv fragments were then screened for binding
15   to a panel of other protein antigens, and were found to
be highly specific. This is illustrated in Fig. 2 with
the single clones with binding activity to human CEA,
MUC1 and human rsCD4. See brief description of figure 2
(supra) for key.

20   Hence, antibody fragments directed against the
human self antigens CEA and MUC1 which are tumour
markers) and rsCD4 can be derived from the same library
and they all have a high specificity for antigen.

Example 4

25   **Characterisation of antiself antibody fragments by DNA**
sequencing and binding to antigen

        The antiself antibody fragments isolated in
examples 1,2 and 3 were characterized by DNA sequencing
and antigen binding.

30

The antibody fragments are derived from a range of
unmutated and somatically mutated V-genes

        The sequences of several clones with
self-specificity were determined as in example 3 and
35   contain both kappa and lambda light chains (Table II).
Comparison with the sequences of the nearest germ-line
V-gene segments indicates that several different
families are used (VH1, 3, 4 and 5; Vk1 and 4, Vl1, 2

and 3). In a few cases the V-genes are completely germline, for example both the VH and Vl genes of aThy-29. However, most of the V-genes have several differences from the nearest germline V-gene segments,
5 both at the nucleotide and amino-acid level (Table II), suggesting that they are derived from somatically mutated B-cells. Some mutations may have arisen during the PCR amplification and assembly process, for example the VH-genes of aFOG1-G8 and aMUC1-1, and the Vk-gene of
10 aThy-33 probably arose from cross-overs between two V-genes during PCR amplification (Table II). Furthermore, large differences (for example the Vk of aFOG1-H6 which differs by 36 nucleotides) may be due to the use of unknown V-gene segments. There is a striking
15 homology in the CDR3 of the heavy chain between aTNF-A1 and aTNF-E1: the germline V-genes are different but the same JH segments are used, and 11/16 residues of CDR3 are identical. This suggests that both scFv fragments may bind to the same epitope of TNF.

20

The antibody fragments are directed to different epitopes on the same protein

The scFv fragments directed against bovine thyroglobulin from example 2 were screened for binding
25 to human thyroglobulin, which differs by only 6 single amino-acid residues in the protomer (Malthiéry,Y. and Lissitzky,S. (1987) Eur. J. Biochem., 165, 491-498). Four of the twelve clones (including aThy-29) bound to human thyroglobulin, whereas the rest (including aThy-32
30 and aThy-33) did not (data not shown). Likewise the fragments binding to the human antibody Fog-1 were screened for binding to a range of other antibodies differing in heavy and light chain isotype (Fig. 3). See brief description of Figure 3 for its key (supra).
35 The fragment aFOG1-A4 bound to all heavy chain g1, 2 and 3 isotypes, but not to g4 or m. By contrast, the fragments aFOG1-H6 and aFOG1-A3 did not bind to any of the other antibodies, including those of the same

isotype as Fog-1, suggesting that they are directed to
the variable domain of Fog-1.

Characterisation of selected scFv fragments

5   The following clones were chosen for large scale
purification and further characterisation: aFOG1-H6,
aFOG1-A3, aTNF-E7, and aThy-29. Colonies of the
non-suppressor E. coli strain HB2151 harbouring the
appropriate phagemid were used to inoculate 2 litres of
10   2 x TY containing 100 μg ampicillin/ml and 0.1% glucose.
The cultures were grown and induced (De Bellis,D. and
Schwartz,I. (1990) Nucleic Acids Res., 18, 1311) and the
tagged scFv fragments purified using the mAb 9E10 as in
(Clackson et al., 1991, supra and WO92/01047).

15   The inhibition of 125I-Fog-1 binding to human Rh D
antigen by the affinity purified scFv fragments aFOG1-H6
and aFOG1-A3 was essentially as performed earlier
(Gorick,B.D., Thompson,K.M., Melamed,M.D. and
Hughes,J.N. (1988) Vox. Sang., 55, 165-170) with the
20   following modifications. 0.0148 μg of 125I-FOG1 was
pre-incubated with varying amounts of purified aFOG1-H6
or aFOG1-A3 scFv fragments (0 - 16 μg) at 37°C for 1.5
hours, before adding 0.5 μl of R1R2 cells (or rr cells
as control). The mixture was then incubated for a
25   further 1.5 hours at 37°C with constant mixing, and
finally cells separated from the supernatant. As a
control, a titration was also performed with a purified
scFv fragment directed against turkey egg white lysozyme
(aTEL9) (Marks et al., 1991, supra).
30   Kinetic measurements were made using surface
plasmon resonance (BIAcore, Pharmacia Biosensor AB)
(Jönsson,U., Fägerstam,L., Ivarsson,B., Lundh,K.,
Löfås,S., Persson,B., Roos,H., Rönnberg,I.,
Sjölander,S., Stenberg,E., Ståhlberg,R., Urbaniczky,C.,
35   Östlin,H. and Malmqvist,M. (1991) BioTechniques, 11,
620-627; Jönsson,U. and Malmqvist,M. (1992) In Turner,A.
(ed.), Real Time Biospecific Interaction. JAI Press
Ltd., San Diego, Vol. 2, pp. 291-336). In order to

separate monomeric and multimeric species, the purified scFv fragments were concentrated by ultrafiltration and then fractionated on a calibrated Superdex 75 FPLC column (Pharmacia) in PBS, 0.2 mM EDTA. Gel filtration

5   was monitored both by the absorbance at 280 nm and on-line to BIAcore with immobilised antigen on the sensor chip (Johnsson et al., 1991).

Kinetic experiments were performed in two different configurations. First, to analyse the binding of

10   soluble scFv, the different antigens were covalently immobilised on the sensor chip (in the case of mAb Fog-1, the antibody was also immobilised via a mouse anti-human kappa light chain mAb using a sensor chip coated with rabbit anti-mouse IgG1). Second, to analyse

15   the binding of the soluble mAb FOG-1, the aFOG1-H6 scFv was immobilised on the chip surface.

The antigens were coupled to the CM5 sensor chip through their amine groups using the Amine Coupling Kit (Pharmacia Biosensor AB)(Johnsson,B., Löfås,S. and

20   Lindqvist,G. (1991) Anal. Biochem., 198, 268-277). The antigens were diluted in 10 mM acetate buffer pH 5.0 to approx. 25 µg/ml, and 3805 resonance units (RU) of TNF, 6249 RU of human thyroglobulin, and 5279 RU of FOG1 were immobilised. For the biospecific presentation of Fog-1,

25   affinity purified rabbit anti-mouse IgG1 (Pharmacia Biosensor AB) was coupled to the surface followed by a mouse mAb anti-human kappa (2300 RU) and then Fog-1 (2050 RU). As binding of the rabbit anti-mouse IgG1 to the mouse mAb was reversible by 10 mM HCl the complex

30   was rebuilt for each analytical cycle. ScFv anti-Fog-1 was coupled to the CM5 surface to 1538 RU. All determinations were performed at 25°C in PBS, 0.2 mM EDTA, 0.05% BIAcore surfactant P20 with a constant flow-rate of 10 µl/min. and an injected volume sample of

35   35 µl. It was not necessary to regenerate the antigen as the scFv fragments rapidly dissociate, with the exception of the biospecific presentation of antigen via rabbit anti-mouse IgG1 which was regenerated with 10 mM

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HCl for 3 min.

Analyses of scFv monomer were performed in the concentration range 100-500 nM, and dimers in the range 40-200 nM except for the biospecifically presented Fog-1
5   where the concentration of dimeric scFv was 0.25-1.26 µM. Fog-1 was analysed on the aFOG1-H6 scFv surface in the concentration range 10-200 nM. All concentrations were calculated from U.V. absorption at 280nm (assuming that 0.7 mg/ml scFv gives an A280 = 1 [Mach,H.,
10   Middaugh,C.R. and Lewis,R.V. (1992) Anal. Biochem., 200, 74-80], and that Mr of a scFv monomer is 30 kD and of a dimer is 60 kD). No correction was made for the fraction of active protein, and therefore the on-rates are an underestimate. The kinetic evaluation of data
15   was performed according to (Karlsson,R., Michaelsson,A. and Mattsson,L. (1991) J. Immunol. Methods, 145, 229-240) and evaluated on the program Origin 1.1 (Microcal inc., Northampton, Mass., USA).

20   Two of the antibody fragments are directed against idiotopes of human mAb Fog-1

The binding of 125I-Fog-1 antibody to human red blood cells bearing the Rh D antigen could be inhibited by both aFOG1-H6 and aFOG1-A3 scFv fragments. Hence,
25   both aFOG1-H6 and aFOG1-A3 are site-associated anti-idiotype antibodies, complexing with the antigen-binding site of Fog-1. The extent of inhibition of 125I-Fog-1 binding to the Rh D antigen (on human R1R2 red blood cells) was determined by titration with
30   affinity purified aFOG1-H6 and aFOG1-A3 scFv fragments. (As control, no inhibition of 125I-Fog-1 binding was observed using a scFv fragment (aTEL9) (Marks et al., 1991, supra) directed against turkey egg white lysozyme). With the maximum of 16 µg scFv (1000 fold
35   molar excess to 125I-Fog-1), the binding was inhibited by 14.2% (aFOG1-H6) and 20.9% (aFOG1-A3), suggesting that the affinities of these fragments for Fog-1 are much lower than the affinity of Fog-1 for the Rh D

antigen ($Ka = 2.2 \times 10^9$ M^{-1}) which binds monovalently (Gorick et al., 1988, supra). If 100% of the fragments are active, the affinities of the two fragments for binding to Fog-1 could be estimated as $Ka = 3 \times 10^5$ M^{-1} for aFOG1-H6 and 6×10^5 M^{-1} for aFOG1-A3, and this is consistent with other kinetic measurements (see below and Table III).

The scFv fragments can form both monomers and dimers in solution

Soluble antibody fragments were purified from bacterial supernatants by affinity chromatography, by binding of the C-terminal peptide tag to the mAb 9E10. After ultrafiltration, the fragments were further purified by FPLC gel filtration (Pharmacia) on Superdex 75 (Pharmacia), and detected on-line both by UV absorption (280 nm) and by binding to antigen immobilised on a sensor chip in BIAcore (Pharmacia Biosensor AB). This showed that the scFv fragments emerged in two peaks, corresponding in size to monomers and dimers. The dimers bind more strongly to the immobilised antigen than monomers due to their greater avidity of binding. The scFv dimers run as monomers on non-reducing SDS gels, and are therefore not linked by disulphide bonds. As two peaks are seen in gel-filtration, it appears that in this case the monomers and dimers do not interconvert rapidly. Presumably the dimers are scFv fragments interlocked through the flexible linker joining the heavy and light chains, or with the heavy chain of one scFv molecule associated with the light chain of the other. We note that antibody Fab fragments made in bacteria can also multimerize (unpublished data).

The scFv fragments have micromolar affinities

The presence of both scFv monomers and dimers could lead to an overestimate of affinity of binding using solid phase methods. To determine the affinity and

kinetics of binding of scFv fragments to the antigen coated chip using surface plasmon resonance, we therefore purified the fragments by gel filtration (Table III). For the dimers, the off-rate constants were

5 determined as about 10^{-2} s^{-1} and the on-rate constants for the scFv dimers as about 10^5-10^6 M^{-1} s^{-1} (assuming the sample is completely active). In the case of aFOG1-H6, the antigen (the mAb Fog-1) was immobilised on the sensor chip in two ways, either directly or via a

10 rabbit anti-mouse IgG1 antibody. The results were almost identical by either method (see Table III). However the active fraction of scFv fragments varies considerably and could lead to an underestimate of the on-rate (and affinity of binding); for example using fluorescence

15 quench titration with several scFv fragments directed against phenyloxazolone we detected only 0.06 to 0.38 functional binding sites per scFv molecule (unpublished data). Indeed the on-rate constants calculated for the association of the aFOG1-H6 fragment and Fog-1 antibody

20 depend on whether the antibody (k_{on} 2.2 x 10^5 M^{-1} s^{-1}) or scFv fragment (k_{on} 1.0 x 10^6 M^{-1} s^{-1}) is immobilised on the sensor chip (Table III), indicating that the aFOG1-H6 fragment is less active than the Fog-1 antibody. For the scFv monomers, the binding signals

25 were low and it was difficult to follow the kinetics of binding to the surface, except for the dissociation of the aThy-29 monomer (k_{off} = 2 x 10^{-2} s^{-1}). However, the four fold stabilisation of the aThy-29 fragment dimer (see below), suggests that the off-rate constants of the

30 other monomers are >10^{-2} s^{-1}, perhaps 10^{-1} s^{-1}.

The greater stability of the scFv dimers on the sensor chip, compared to monomers, indicates that the dimers are bivalent. The scFv dimers are therefore analogous to the two heads of the antibody IgG (but with

35 different spacing between the heads), and their binding avidities were estimated as about 10^7 M^{-1} from k_{on}/k_{off} (Table III). The affinities of the monomers must be lower by virtue of their faster dissociation from the

surface. For the aThy-29 monomer, and assuming that the on-rate constant is the same as for the dimer (Mason,D.W. and Williams,A.F. (1986) Kinetics of Antibody Reactions and the Analysis of Cell Surface
5   Antigens. Blackwell Scientific, Oxford), we can estimate an affinity of about 3×10^6 M^{-1}. These affinities, calculated from the rate constants measured by surface plasmon resonance appear to be similar to those measured in solution by fluorescence quench
10   techniques. For example the affinity of binding of the monomer scFv fragment aTEL9 (Marks et al., 1991) which binds to turkey lysozyme (and was derived from the same library) was estimated as 3.9×10^7 M^{-1} using surface plasmon resonance (Table III), and as 1.2×10^7 M^{-1} by
15   fluorescence quench (Marks et al., 1991, supra).
The affinities of antibodies isolated are typical of antibodies from the mouse primary immune response (Foote,J. and Milstein,C. (1991) Nature, 352, 530-532). The kinetics of association of the antibody fragments to
20   the protein self-antigens (10^5 to 10^6 M^{-1} s^{-1}) are also typical of previously characterised Ab-protein interactions. However the kinetics of dissociation (10^{-2} s^{-1}) are relatively fast for Ab-protein interactions (but both rates are slow compared to many
25   Ab-hapten interactions). At first sight, it is surprising that we can isolate scFv fragments with such fast off-rates, as one would not expect a "monomeric" phage to be retained on the solid support during washing. However, scFv fragments are displayed
30   multivalently on the phage, especially using the M13DgIII helper phage, and some of the scFvs which tend to form dimers in solution, may also form dimers on phage. The multivalent interactions with antigen help retain the phage, allowing the encoded scFv phage to be
35   isolated.
Random combinatorial V-gene repertoires derived from the mRNA of immunised animals are enriched for heavy or light chain V-genes encoding part of an antigen

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binding site and this facilitates the isolation of
antigen-binding fragments using phage technology,
although the combinations of V-genes of each
B-lymphocyte appear to be largely destroyed. Antigen
5   binding sites can also be generated de novo by the
random combination of chains, as illustrated by the
isolation of scFv fragments against foreign antigens
from unimmunised human donors (Marks et al., 1991,
supra).

10   "Natural autoantibodies", self-reactive antibodies
isolated from healthy donors tend to be of low affinity
and polyspecific and may well be produced by a discrete
subset of B-cells, the internal activity set
(Holmberg,D. and Coutinho,A. (1985) Immunol. Today, 6,
15   356-357), contributed in part by CD5+ B-cells (Casali,P.
and Notkins,A.L. (1989) Annu. Rev. Immunol., 7,
513-535). In contrast, the anti-self scFv fragments we
have made are highly specific in binding to antigen
despite only having micromolar affinities. This is a
20   surprising and valuable finding. Their affinities could
presumably be improved in vitro, for example, the
affinity of an scFv fragment for the hapten
phenyloxazolone derived from the phage library (and,
like the anti-self antibodies described here, with a
25   relatively fast off-rate) was improved from $Ka = 3.1 \times 10^6 \, M^{-1}$ to $9.1 \times 10^8 \, M^{-1}$ by chain shuffling (WO92/01047;
Marks et al., 1992b, Biotechnology 10, 779-783, 1992).
This would allow the creation of highly specific, high
affinity human antibodies directed against self-antigens
30   for use in human therapy.

Example 5:
Creation of a Synthetic Library

By display of antibody repertoires on the surface
35   of filamentous phage and selection of the phage with
antigen[1], we can mimic immune selection [2,3] and make
human antibodies from the rearranged V-genes of
unimmunised donors[4]. Human antibodies have now been

made by synthesis from defined V-gene elements. A repertoire of 49 human germ line V_H gene segments was rearranged in vitro by joining to a synthetic "D-segment" of five random amino acid residues and a J-segment, to create a synthetic third complementarity determining region (CDR) of eight residues. The rearranged V_H genes were cloned with a human Vlambda3 light chain as single-chain Fv fragments for phage display. The library of 10^7 phages was panned with a hapten 2-phenyl-oxazol-5-one (phOx) conjugate to bovine serum albumin (BSA), and phage isolated that encoded fragments with specific binding activity to phOx-BSA, and with affinities to phOx-gamma-aminobutyric acid (phOx-GABA) in the micromolar range. Comparison of twenty one clones with unique sequences showed that the in vitro "immune response" to the hapten was largely restricted to the V_H26 segment (V_H3 family)[6] with an invariant aromatic residue (Tyr, Phe, Trp) at residue 98 of CDR3. The use of V-genes rearranged in vitro may allow the design of antibody libraries biased towards the binding of antigens of known structure, and the creation of therapeutic human antibodies with reduced immunogenicity.

Antibody variable domains consist of a β-sheet framework with three loops of hypervariable sequence or CDRs[5]. The loops create antigen binding sites of a variety of shapes, ranging from flat surfaces[7] to pockets[8]. For human heavy chains, the sequence diversity of the first two CDRs are encoded by a repertoire of about fifty germ line V_H segments. (I.M. Tomlinson et al., supra). The third CDR is generated from the recombination of these segments with about thirty D and six J segments[9], and although its sequence is highly variable, it often includes a salt bridge from Asp101 of the loop to Arg94 of the framework[10]. The structures and lengths of the first two CDRs are restricted[10,11], but those of CDR3 differ greatly, with lengths ranging from 4 to 25 residues[5].

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A library was created of rearranged V_H genes with a CDR3 of eight residues including Asp101, in combination with a single Vlambda (ref.12) light chain. Forty nine germ line V_H segments encoding most of the human V_H repertoire (Tomlinson et al., supra) were each amplified using the polymerase chain reaction[13] and oligonucleotide primers that introduce a synthetic D-segment (of 15 bases of random sequence at the 3' end of the V_H segment) and a J-segment, together encoding a CDR3 loop of eight residues (Fig.4). The rearranged segments were pooled and cloned for phage display with a human Vlambda3 light chain, creating a synthetic library of 10^7 phage clones. Like the immune system, the synthetic library of 10^7 phage clones can tap only a small fraction of the potential diversity. Thus the diversity is potentially $49 \times 32^5 = 1.6 \times 10^9$ different nucleotide sequences, or $49 \times 20^5 = 1.6 \times 10^8$ different amino acid sequences.

The library was subjected to four rounds of growth and panning on phOx-bovine serum albumin (BSA) coated tubes, and clones screened as soluble[14] single chain Fv fragments[15,16] for binding activity to phOx-BSA by ELISA[4]. After the third and fourth rounds, 14/96 and 61/96 clones respectively were identified with binding activities to phOx-BSA and of these (29 tested) none bound to other proteins (see legend Table B). Furthermore their binding to phOx-BSA coated plates could be competed with the soluble hapten (Table B).

Sequencing revealed that many (21/29) of the phOx binders were unique, with an eight residue CDR3, and utilised either a segment from the V_H4 family, or one of three segments from the V_H3 family (Table B). Together these segments use three of the seven "canonical" folds available to the first two hypervariable loops of human V_H segments. (C. Chothia, et al., supra). The majority of the unique clones (16/21) were derived from the VH26 segment[6] and have related sequences in the third hypervariable loop: in this group the first residue

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tends to have a branched aliphatic side chain (15/16), the second residue tends to be lysine or arginine (11/16), while the fourth residue is always an aromatic residue (most frequently tyrosine).

5   The affinities (K_d) of two of the stronger binders (Ox 13 and Ox-31, Table B) for phOx-GABA were determined by fluorescence quench titration[17] as 3.1 ± 0.2 µM and 6.7 ± 0.7 µM respectively. Although the synthetic antibody library lacks the diverse VH-CDR3 lengths and

10   the different light chains of antibodies made _in vivo_, the affinities for phOx-GABA compare with 0.5 µM for a (phage) antibody made from unimmunised human donors[4], or 1µM for several hybridomas from a mouse primary immune response[18] (but see caveat, Table A legend). To improve

15   these affinities, one could systematically alter (see below) the many different phOx antibodies selected (Table A).

       In principle, the use of phage display libraries of V-genes rearranged _in vitro_ offers an attractive

20   alternative to those rearranged _in vivo_[4]. Firstly the framework regions and first two hypervariable loops of both heavy and light chains of the synthetic human antibodies created from the library are essentially germ line. This contrasts with the "primary" phage

25   antibodies tapped from human V-genes rearranged _in vivo_, in which the extent of somatic mutation varied widely[4]. Leaving aside polymorphism, the VH gene segments are identical in different individuals, and the synthetic antibodies are potentially less immunogenic. By

30   altering the lengths and sequences of the heavy and light chain CDR3 loops, or by localising the minimal mutations in the other CDR loops, or by shuffling with synthetic "germ line" light chains[19,20], it may be possible to improve their affinities while retaining

35   their germ line character.

       Secondly both kinds of libraries are highly biased. In the "natural" libraries, the bias is outside our control, and is imposed for example by allelic

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variation, deletion polymorphism and deletion of self-
reactive clones. In the synthetic library, the bias can
be introduced systematically. Here for example, all the
VH-gene segments, were chosen and thereby the folding of
5   the first and second hypervariable loops: also fixed
were the length and diversity of VH-CDR3 and the light
chain. Although several ways of making diverse
synthetic libraries have been suggested[2], it should also
be possible to incorporate design principles into the
10   encoded structures. If the shape of the antigen were
known, an envelope of roughly complementary binding
sites might be designed and built with defined V-gene
elements. Use of such "designer" libraries would favour
the isolation of antibodies with higher affinities.

15

Table A

Family	No. of genes	VH segments*	Library size $\times 10^{-6}$ (%)
V_H1	14	1-5,7,8,10,12, 14,15,20,21,25	2.3 (20)
V_H2	1	27	1.0 (9)
V_H3	23	29-33,35,38-40, 42,44-54,58,59	2.1 (19)
V_H4	9	63-71	2.6 (23)
V_H5	1	73	1.4 (12)
V_H6	1	74	1.9 (17)
Total:	49		11.3 (100)

----

*for simplicity V_H segments are listed according to
DP nomenclature of Tomlinson et al., supra.

Table A - Composition of the synthetic library

Forty nine human V_H segments (Tomlinson et al,
supra) were used, one for each of the V_H2, V_H5 and V_H6
gene families and multiple segments for the other three
families, and cloned according to family. Clones from
the V_H segments of each family were checked for presence
of insert (on average 85%) and pooled into a single
large library as in Table B, creating a (controlled)
bias for certain gene families. The segments from the
V_H2, V_H5, V_H6 families are thereby "overrepresented"
with respect to the segments from other families.
Sequencing of thirty five clones from the unselected
library confirmed that V_H segments from each family were
present, and that the nucleotides were present in the
expected ratios in the D-segment, but with a slight bias
for C. (At the first and second position of each codon,
A, 21.3%; G, 17.9%; C33.7% and T, 27.1%; at the third
position, G, 42.6% and T, 57.4%). The expression levels
of the antibody fragments were also checked, and V_H
segments were identified in clones with detectable
expression levels, for example V_H1 (DP-7), V_H2 (DP-27),
V_H3 (DP-29,35,38,44,47,51,53), V_H4 (DP-63,69), V_H5 (DP-
73) and V_H6 (DP-74).

Methods

The clones were checked for presence of insert by
'PCR-screening'[21] with oligonucleotides LMB3 and pHEN-
SEQ (ref.4) and sequenced from double-stranded DNA by
the dideoxy chain termination method[22] with
oligonucleotide LINKSEQ (5'-CGA TCC GCC ACC GCC AGA G-
3'). (The numbers in the tables are corrected for
insert). Expression of soluble scFv fragments was
checked by spotting 10μl supernatant of induced
overnight cultures in E. coli HB2151 (ref.14) onto a
nitrocellulose filter using a slot-blot device (Minifold
II, Schleicher and Schuell), and detecting the bound
peptide-tagged scFv fragments with 9E10 antibody[23] and

peroxidase labelled anti-mouse antibodies (Sigma).

Table B

Clone	Family	Germline gene*	Canonical Loop structure*	I_{50}^{ϕ}
Ox-31	V_H3	DP-42	1-1	26
Ox-15	V_H3	DP-45	1-1	>300
Ox-18	"	"	"	>300
Ox-33	V_H3	DP-47	1-3	20
Ox-13	"	"	"	50
Ox-9	"	"	"	80
Ox-7	"	"	"	86
Ox-30	"	"	"	86
Ox-12	"	"	"	86
Ox-5	"	"	"	100
Ox-3	"	"	"	125
Ox-20	"	"	"	125
Ox-21	"	"	"	125
Ox-4	"	"	"	130
Ox-10	"	"	"	150
Ox-14	"	"	"	180
Ox-19	"	"	"	250
Ox-25	"	"	"	>400
Ox-27	"	"	"	¶
Ox-2§	V_H4	DP-67	2-1	>400
Ox-1	"	"	"	>400

* Tomlinson et al., supra, Chothia et al., supra.

ϕ in µM, according to competition ELISA with phOx-GABA.

§ shows V67A mutation in FR3.

¶ Not determined.

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Table B - phOx-binders isolated from the synthetic library

Phage were prepared from the library by rescue with VCS-
M13, and subjected to rounds of panning in phOx-BSA
coated tubes as in ref.4. The sequences of 21 phage
binding to phOx revealed four germ line VH segments, DP-
42,45,47 (VH3 family) and DP-67 (VH4 family). DP-47 is
identical to VH26 (ref.6, corrected in ref.24), while
DP-42, DP-45 and DP-67 only differ in one or a few
framework residues from 8-1B (ref.25), 65-2 (ref.26) or
VH4.22 (ref.27) respectively. Clones from the
unselected library using the DP47 V_H segment and lacking
the characteristic pattern of CDR3 did not bind to phOx.
Of the 21 phOx binders tested, none bound to BSA, NIP-
BSA, plastic, chymotrypsinogen A, cytochrome c, bovine
thyroglobulin, keyhole limpet haemocyanin or turkey egg
white lysozyme. Four clones that bound to BSA (but not
to phOx) were found to be contaminants (αBSA3 clones,
from ref.4).

Methods

As in ref.4. The relative affinities of the scFv
fragments were determined by inhibition ELISA[28]. A
serial dilution of 4-gamma-amino-butyric acid methylene
2-phenyl-oxazol-5-one (phOx-GABA), with concentrations
ranging from 6 to 400μM, was made in 4% Marvel-PBS, and
scFv supernatant added. The concentration of phOx-GABA
resulting in a 50% reduction of the signal (I_{50}) for
binding to phOx-BSA was noted. The affinities of the
clones Ox-13 and Ox-31 for phOx-GABA were determined by
fluorescence quench titration using scFv purified by the
c-myc tag (ref.4). Ideally, the affinity for the phOx-
BSA conjugate would have been measured directly, or that
for phOx-caproic acid, but phOx-GABA was used here to
allow comparison with the hybridoma data of ref.18. The
affinities of the antibodies for the phOx conjugate, or
for phOx-caproic acid are likely to be better than those

65

measured for phOx-GABA.

Figure 4 - Shows the assembly of rearranged VH genes
(see text)

5

Methods

     A synthetic oligonucleotide SYNLIB1 (see Table IV)
introduced a D-segment with a five residue random amino
acid sequence, a J-segment and an XhoI restriction site,
10 to the 3' end of each of 49 human V_H germline segments
(Tomlinson et al., supra). The primer was used in the
polymerase chain reaction[13] with a V_H family based back
primers (VHBACK) incorporating an NcoI site[4],
HuVH1BackSfi to HuVH6BackSfi. Each V_H segment clone
15 (provided as single stranded template in M13 vector) was
amplified separately at 94°C for 1 min, 50°C for 1 min,
and 72°C for 1.5 min, for 25 cycles, on a PHC-3
thermocycler (Techne). Each amplification was checked
by electrophoresis on agarose gel, and similar amounts
20 of DNA from V_H segments of the same family were pooled,
digested with NcoI and XhoI, and cloned into the vector
pHEN1 (ref.14) carrying a rearranged Vlambda3 light
chain variable domain (IGLV3S1; ref.12) taken from a
scFv fragment binding to BSA[4].

25   If, instead of a random oligonucleotide, an
oligonucleotide encoding a CDR, eg from a rodent, were
used, this would imprint that non-human CDR on the
product synthetic human library.

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Example 6
Isolation of antibody fragments specific for tumour necrosis factor-a from a germ line human synthetic library

A clone encoding an antibody fragment specific for tumour necrosis factor-α was isolated from a germ line human synthetic library. This library was prepared as described in example 5, except that the oligonucleotide SYNLIB2 was used in place of SYNLIB1, so that a 5 amino acid V_H CDR3 was generated. The library was panned against tumour necrosis factor-α, as described in example 1 for the library derived from unimmunised humans. After four rounds of panning a phage antibody (and corresponding soluble fragment) was isolated with binding activity to TNF. The V_H region of the scFv fragment (αTNF-10) was derived from the VH segment DP-45 (Tomlinson et al, 1992, supra). The hapten binding clones αNIP-6, αNIP-12, αOx-15 and αOx-18 are also derived from this segment, although each of these fragments were nevertheless specific for binding to hapten or TNF. This indicates that antigen binding sites with entirely different specificities can be created on the same antibody framework by substitution of CDR3 alone. Binding to non-specific antigens was assessed by ELISA as described in example 1.

Example 7

Isolation of single chain Fv fragments binding to human thyroglobulin and a human monoclonal antibody from a germ line human synthetic library containing VH CDR3 sequences of different lengths

A germ line human synthetic single chain Fv fragment library was prepared in an analagous manner to the library in Example 5, to include germ line VH segments and synthetic DH and JH regions, generating VH CDR3 regions of between 4 and 12 amino acids. A single germ line rearranged light chain was provided. This phage library has been used as a source of antibody fragments with anti-human specificities.

Fifty germ line gene VH segments (Tomlinson et al, 1991 supra, as in Example 5) were amplified with oligonucleotides to introduce a completely randomised CDR3 varying in length from 4 to 12 residues. In a first PCR-reaction, each gene was amplified with its family specific VHBACK-primer (one of VH1BACKSfi to VH6BACKSfi; Marks et al, 1991 supra; WO92/01047) at the 5' end, and, annealing at the 3' end, one of each of the oligonucleotides of the series SYNLIB4 – SYNLIB12 (Table IV). The PCR contained 2.5 pmol of each of the appropriate pair of oligonucleotides per 50 µl reaction mix containing 250µM dNTPs, 10mM KCl, 10mM $(NH4)_2SO_4$, 20mM TrisHCl (pH8.8), 2mM MgCl2, 100µg/ml BSA and 1µl (1 unit) of Taq DNA polymerase (Cetus). The template was 1µl of a bacterial stock of E.coli infected with a M13 phage clone encoding the appropriate germ line V gene. The amplification cycle was 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min. After 25 cycles, 30 pmol of the same VHBACK oligonucleotide and 30pmol of JHSAL (Table IV) was added, and the PCR continued for 15 cycles, introducing a SalI cloning site at the 3' end of the VH-gene. After verifying that a band of the appropriate size was seen on agarose gel electrophoresis, the PCR products of all amplifications done with the same SYNLIB primer were collected, cut

with <u>Nco</u>I and <u>Sal</u>I, and cloned into <u>Nco</u>I-<u>Xho</u>I-cut pHEN1-
V 3 (pHEN1 containing cloned IGLV3S1) as in Example 5.
In this way, 9 libraries (each with one particular CDR3
length) were made, each containing between 5×10^6 and 5×10^7 clones.

5

Selection

Phage was prepared from the nine different
libraries by rescue with VCS-M13 as described in Example
10   3. Phage from the nine individual libraries was mixed
to give one large library and subjected to panning on
one of each of 2 antigens: Immunosorp tubes were coated
with OAK3 (human anti-Rhesus D antibody, IgG3, k)
overnight in carbonate buffer (0.1 M NaHCO$_3$, pH 9.6 at
15   100 µg/ml) or human thyroglobulin (coated at 100µg/ml in
PBS). Selections were performed as in Example 3.

Screening

ELISA was performed as described in Hoogenboom et
20   al, 1991 supra. ELISA plates were coated overnight with
OAK3 at 100µg/ml in PBS at room temperature or with
human thyroglobulin at 100µg/ml at room temperature.

Results

25   After four rounds of selection on OAK3-coated
tubes, eluted phage was used to infect HB2151, and
soluble scFv fragments analysed for binding by ELISA.
59/96 clones were scored positive in the OAK3 ELISA.
The germ line human synthetic library was also
30   subjected to: 5 rounds of selection on human
thyroglobulin coated tubes, 80/96 clones were found to
be positive in a phage ELISA of individual clones
rescued with VCS-M13.
Two of each of the positive clones were analysed in
35   ELISA for binding against a range of antigens (OAK3,
human thyroglobulin, phOx-BSA, NIP-BSA, BSA, ovalbumin,
chymotrypsinogen-A, streptavidin, cytochrome c, KLH,
turkey egg-white lysozyme). The two OAK-3-binding

clones (as soluble scFv fragments) both gave signals approximately 3-fold higher than background in ELISA on OAK3. The two thyroglobulin binding clones (as scFv fragments displayed on phage) both gave signals approximately 5-fold higher than background in thyroglobulin-ELISA. All the clones were found to be highly specific for the antigen against which they had been selected. By hybridisation to family-specific primers (J.D. Marks et al, Eur. J. Immunol. 21 985-991 1991), the VH segment of all four clones was identified to be of the VH3 family. The CDR3 length of each clone was analysed by amplifying the CDR3 with oligonucleotides CDRFOR and CDRBACK (Table IV), and analysing the product on a 8% polyacrylamide gel. For the two OAK3-binding clones, we found a length of 4 or 7 amino acid residues, while the thyroglobulin binding clones both use a CDR3 length of 10 residues.

Hence, antibody scFv fragments binding to a human monoclonal antibody and a human self antigen have been isolated from a human germ line synthetic library.

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Example 8

Isolation of antibody fragments triggering the activity of the interleukin-1 receptor

The library of single chain Fv fragments derived
5   from an unimmunised human that was described in Example
1 is used to select antibodies which will trigger the
activity of the interleukin-1 receptor. Antibody
fragments are first isolated which bind to the soluble
external domain of the interleukin-1 receptor (IL-1R) of
10 T cells. Antibody clones that are thus identified are
then analysed in assays for interleukin-1 type
biological activity. The IL-1R on murine and human T
cells is a highly homologous 80kD cell surface
glycoprotein which binds both interleukin-1α and
15 interleukin-1β. A cDNA clone encoding the N terminal
316 amino acids of the murine receptor external domain
has been expressed in HeLa cells (S.K. Dower et al. J.
Immunol. 142 4314-4320 1989). The soluble IL1-R
molecule thus expressed has been purified and shows
20 binding properties indistinguishable from the full
length IL-1R molecule, a complex being formed between a
single soluble IL1-R molecule and IL-1. This soluble
receptor molecule binds to human interleukin-1. The
human T cell interleukin 1 receptor has been cloned and
25 sequenced by J.E. Sims et al (Proc. Natl. Acad. Sci. USA
86 8946-8950, 1989). The soluble external domain of the
human IL1 receptor, amino acids 1 to 316, is expressed
in HeLa cells and purified as described for the murine
receptor.
30   The rescued unimmunised human library is first
selected against the recombinant human soluble IL-1
receptor, corresponding to the external domain of the
IL-1 receptor. Immunotubes are coated with the soluble
IL-1 receptor as described in Example 1 at 10μg/ml and
35 panning is performed as described in Example 1 for a
total of four rounds of affinity selection.
          Clones binding to soluble IL-1 receptor are
characterised by ELISA using microtitre plates coated

with recombinant soluble IL-1 receptor at 10µg/ml as
described for TNF-α in Example 3. Antibody fragments
showing significant ELISA signals with soluble IL-1
receptor but not with non-specific antigens are then
5   chosen for further study.

Antibody clones isolated in this way are then
expressed as soluble scFv fragments in E.Coli and
purified as described in Example 4 by mAb 9E10 affinity
chromatography. Binding to human receptors is assessed
10   using binding of ^{125}I-labelled antibody fragment to
human fibroblast cell line TIG-1 expressing the
interleukin-1 receptor basically as described by T.Takii
et al (Eur. J. Immunol. 22 1221-1227 1992) for
determining the affinity of ^{125}I-IL1α for the receptor
15   on these cell lines. The purified antibody fragments
that show receptor binding are used in a biological
screening assay using human epithelial cells to examine
them for stimulation of synthesis of prostacyclin (PGI2)
and platelet activating factor (PAF) as described by E.
20   Dejana et al (Blood 69 695-699, 1987). These studies
will identify antibody fragments which have an antiself
specificity against
IL-1 receptor which triggers receptor activity. The
activity can be quantified relative to human
25   interleukin-1α using a standard bioassay for IL-1α for
example proliferation of the D10S helper T cell line
using ^{3}H-thymidine incorporation (S.F. Orencole and C.A.
Dinarello Cytokine 1 14-22 1989) or a conversion
proliferation assay as described by A.J. Gearing et al
30   (J. Immunol. Methods 99 7-11, 1987).

Table I   Frequency of binding clones isolated from the
               unimmunised scFv library after selection

Antigen	Rounds of selection					No. of unique clones
	1	2	3	4	5	
Thyroglobulin (bovine)	−	−	18/40	−	−	12
Thyroglobulin (human): selected on bovine	−	−	10/40	−	−	4
Fog1 (human IgG1, k antibody)	−	−	−	94/96	−	4
TNFα (human)	−	122/1920	83/192	92/96	−	7
CEA (human)	−	−	0/96	1/96	2/96	1
MUC1 (human): selected with peptide	−	−	−	0/96	2/96	1
rsCD4 (human)	−	−	−	−	8/96	1

The ratios indicate the frequency of binding clones after each
round of selection.   Phagemids were rescued with M13DgIII
helper phage, except for the CEA, MUC1 and rsCD4 selections,
where VCS-M13 helper phage was used.

Table II V-gene family, germline derivation and extent of somatic hypermutation of several antigen-specific scFv fragments isolated from the unimmunised library

scFv	Family	Germline gene of closest nucleotide sequence	Differences from germline	
			Nucleotide	Amino-acid
HEAVY CHAINS				
αThy-23	VH3	DP-47	13	8
αThy-29	VH1	DP-14	0	0
αThy-32	VH3	DP-31	5	2
αThy-33	VH3	DP-49	32	19
αFOG1-A3	VH3	DP-54	7	3
αFOG1-A4	VH3	DP-46	7	7
αFOG1-H6	VH3	DP-51	10	4
αFOG1-G8[a]	VH4	DP-63(FR1)	2	0
	VH5	DP-73(CDR1 to FR3)	15	7
αTNF-A1	VH3	DP-50	9	6
αTNF-E1	VH3	DP-46	14	6
αTNF-E7	VH1	DP-10	0	0
αTNF-H9G1	VH1	DP-4	1	1
αCEA4-8A	VH1	DP-14	1	0
αMUC1-1[a]	VH1	VI-2 (FR1 to CDR2)	2	0
	VH1	DP-25(FR3)	0	0
αCD4-74	VH5	DP-73	13	8
LIGHT CHAINS				
αThy-23	Vk1	L8	20	9
αThy-29	Vλ3	IGLV3S1	0	0
αThy-32	Vλ1	IGLV1S2	1	1
αThy-33[a]	Vk1	L12(FR1 & CDR1)	6	3
	Vk4	B3(FR2 to FR3)	5	5
αFOG1-A3	Vλ2	VL2.1	16	9
αFOG1-A4	Vk1	O4	25	12
αFOG1-H6	Vk1	L5	36	17
αFOG1-G8	Vk1	L8	27	14

scFv	Family	Germline gene of closest nucleotide sequence	Differences from germline	
			Nucleotide	Amino-acid

LIGHT CHAINS

scFv	Family	Germline gene of closest nucleotide sequence	Nucleotide	Amino-acid
αTNF-A1	Vk1	L11	12	8
αTNF-E1	Vk1	L5	5	5
αTNF-E7	Vk1	L11	17	8
αTNF-H9G1	V 1	IGLV1S2	18	9
αCEA4-8A	Vk1	O2	4	0
αMUC1-1	V 2	VL2.1	18	12
αCD4-74	V 1	Humlv1L1	23	17

References for all the heavy chain germline genes can be found in Tomlinson et al. (1992). The references for the light chains are VL2.1 (Brockly et al. 1989); IGLV1S2 (Bernard et al. 1990); IGLV3S1 (Frippiat et al. 1990); L8(Vd) and L5(Vb) (Pech et al., 1984); L12(HK102) (Bentley and Rabbits, 1980); B3(VKIV) (Klobeck et al., 1985); O2 and O4 (Pargent et al., 1991); L11 (Scott et al., 1991); Humlv1L1 (Daley et al., 1992). Alternative names are given in parenthesis.

a)   These genes appear to have been created by cross-overs between two V-genes during PCR amplification and therefore matches have been determined using the two putative germline segments: FR, framework; CDR, complementarity-determining region.

Bentley, D.L. and Rabbits, T.H. (1980) Nature, 288, 730-3.

Bernard, F., Chuchana, P., Frippiat, J.P., Buluwela, L. and Lefranc, M.P. (1990) Nucleic Acids Res, 18, 7139.

Brockly, F., Alexandre, D., Chuchana, P., Huck, S., Lefranc, G. and Lefranc, M.P. (1989) Nucleic Acids Res, 17, 3976.

Frippiat, J.P., Chuchana, P., Bernard, F., Buluwela, L., Lefranc, G. and Lefranc, M.P. (1990) Nucleic Acids Res, 18, 7134.

Klobeck, H.G. Bornkamm, G.W., Combriato, G., Mocikat, R.,

Pohlenz, H.D. and Zachau, H.G. (1985) Nucleic Acids Res, 13, 6515-29.

Pargent, W., Meindl, A., Thiebe, R., Mitzel, S. and Zachau, H.G. (1991) Eur J Immunol, 21, 1821-7.

Pech, M., Jaenichen, H.R., Pohlenz, H.D., Neumaier, P.S., Klobeck, H.G. and Zachau, (1984) J Mol Biol, 176, 189-204.

Scott, M.G., Crimmins, D.L., McCourt, D.W., Chung, G., Schable, K.F., Thiebe, R., Quenzel, E.M., Zachau, H.G. and Nahm, M.H. (1991) J Immunol, 47, 4007-13.

Tomlinson, I.M. Walter, G., Marks, J.D., Llewelyn, M.B. and Winter, G. (1992) J.Mol.Biol., 227, in press.

Table III Affinities and kinetics of antigen binding by monomeric and dimeric scFv fragments

scFv	(M/D)[a]	Immobilised species	k_{on}[b] (BIAcore) M^{-1} s^{-1}/10^4	k_{off}[b] (BIAcore) s^{-1}/10^{-2}	$K_a = k_{on}/k_{off}$ (BIAcore) M^{-1}/10^6	K_a' by FQ[c]) or inhibition[d] M^{-1}/10^6
αTNF-E7	D	Human TNFα	9.0 (±1.2)	1.4 (0.054)	6.4	ND
αFOG1-H6	D	Fog-1 (direct)	22.2 (±0.4)	1.8 (0.23)	12.3	ND
αFOG1-H6	D	Fog-1 (via RAMIgG1)	22.1 (±1.9)	2.4 (0.045)	9.3	ND
αFOG1-H6	D	αFOG1-H6 scFv	104 (±2.4)	ND[c]	ND	ND
αFOG1-H6	M+D	(Measured by inhibition)	ND	ND	ND	0.3[d]
αFOG1-A3	M+D	(Measured by inhibition)	ND	ND	ND	0.6[d]
αThy-29	D	Human Thyroglobulin	6.6 (±1.2)	0.46 (0.063)	14.3	ND
αThy-29	M	Human Thyroglobulin	ND	2.0 (0.37)	ND	ND
αTEL9	M	Turkey Egg Lysozyne	39.2 (±2.6)	1.0 (0.97)	39.2	11.6[e]

a) M, monomeric fraction; D, dimeric fraction b) Numbers in brackets are standard deviations c) FQ, fluorescence quench titration e) Not determined because the dissociation

d) Calculated from the extent of inhibition of ^{125}I-Fog-1 binding to the Rh D antigen

curves were very badly bent

Table IV Oligonucleotides used

SYNLIB1 :   5'GCC TCC ACC TCT CGA GAC GGT GAC CAG GGT ACC TTG
            GCC CCA ATA GTC AAA (A/CNN)5 TCT TGC ACA GTA ATA
            CAC GGC CGT GTC-3'

SYNLIB2 :   5'GCC TCC ACC TCT CGA GAC GGT GAC CAG GGT ACC TTG
            GCC CCA (A/CNN)5 TCT TGC ACA GTA ATA CAC GGC CGT
            GTC-3'

SYNLIB4 :   5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)4 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

SYNLIB5 :   5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)5 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

SYNLIB6 :   5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)6 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

SYNLIB7 :   5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)7 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

SYNLIB8 :   5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)8 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

SYNLIB9 :   5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)9 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

SYNLIB10 : 5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)10 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

SYNLIB11 : 5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)11 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

SYNLIB12 : 5'-GAC CAG GGT ACC TTG GCC CCA ((A/C)NN)12 TCT TGC
            ACA GTA ATA CAC GGC CGT GTC-3'

JHSAL :   5'- GCC TGA ACC GCC TCC ACC AGT CGA CAC GGT GAC
            CAG GGT ACC TTG GCC CCA-3'

CDRFOR :   5'- CAG GGT ACC TTG GCC CCA-3'

CDRBACK :   5'- GTG TAT TAC TGT GCA AGA-3'

Human VH Back Primers

HuVH1aBACKSfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG
               GTG CAG CTG GTG CAG TCT GG-3'

HuVH2aBACKSfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG
               GTC AAC TTA AGG GAG TCT GG-3'

HuVH3aBACKSfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG
               GTG CAG CTG GTG GAG TCT GG-3'

HuVH4aBACKSfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG
               GTG CAG CTG CAG GAG TCG GG-3'

HuVH5aBACKSfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG
               GTG CAG CTG TTG CAG TCT GC-3'

HuVH6aBACKSfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG
               GTA CAG CTG CAG CAG TCA GG-3'

CLAIMS:

1.   A method of obtaining a member of a specific binding pair (sbp member), the sbp member being an antibody or antibody fragment and having an antigen binding site with binding specificity for an antigen which is a self antigen of a species of mammal, the method comprising:

(a)   providing a library of replicable genetic display packages (rgdps), each rgdp displaying at its surface an sbp member, and each rgdp containing nucleic acid with sequence derived from said species of mammal and encoding a polypeptide chain which is a component part of the sbp member displayed at the surface of that rgdp;

(b)   selecting, by binding with said self antigen, one or more sbp members with binding specificity for said self antigen.

2.   A method according to claim 1 wherein said providing a library of rgdps comprises:

combining (i) a first polypeptide chain component part of an sbp member fused to a component of a rgdp which thereby displays said first polypeptide chain component part or population thereof at the surface of rgdps on expression in a recombinant host cell organism, or a population of such a first polypeptide chain component part fused to a said component of a rgdp, with (ii) a second polypeptide chain component part of an sbp member or a population of such a second polypeptide chain component part, to form a library of sbp members displayed at the surface of rgdps;

at least one of said first or second polypeptide chain component part or populations thereof being encoded by nucleic acid which is capable of being packaged using said component of an rgdp.

3.   A method according to claim 1 werein said providing a library of rgdps comprises:

combining (i) nucleic acid which encodes a first polypeptide chain component of an sbp member fused to a component of a rgdp or a population of such a first

polypeptide chain component part fused to a component of a rgdp, with (ii) nucleic acid encoding a second polypeptide chain component part of an sbp member or a population thereof, to form a library of nucleic acid,

5 nucleic acid of said library being capable of being packaged using said component of an rgdp;

expressing in a recombinant host organism said first polypeptide chain component part fused to a component of a rgdp or population thereof and said second polypeptide

10 chain component part of an sbp member or a population thereof, to produce a library of rgdps each displaying at its surface an sbp member and containing nucleic acid encoding a first and a second polypeptide chain component part of the sbp member displayed at its surface.

15 4. A method according to claim 1, 2 or 3 wherein each said sbp member displayed at the surface of an rgdp is an antibody fragment comprising a V_H domain and a V_L domain.

5. A method according to claim 2 wherein both said first and second polypeptide chain component parts or

20 populations thereof are expressed from nucleic acid capable of being packaged using said component of an rgdp.

6. A method according to any preceding claim wherein each said sbp member displayed at the surface of an rgdp

25 is an scFv antibody fragment.

7. A method according to claim 2 or claim 3 wherein said second polypeptide chain component part or population thereof is encoded by nucleic acid separate from nucleic acid encoding said first polypeptide chain

30 component part or population.

8. A method according to claim 1 or claim 7 wherein each said sbp member displayed at the surface of an rgdp is an Fab antibody fragment.

9. A method according to any one of the preceding

35 claims wherein the nucleic acid is derived from rearranged V genes of an unimmunised mammal.

10. A method according to any one of claims 1 to 8 wherein the nucleic acid is derived from a library
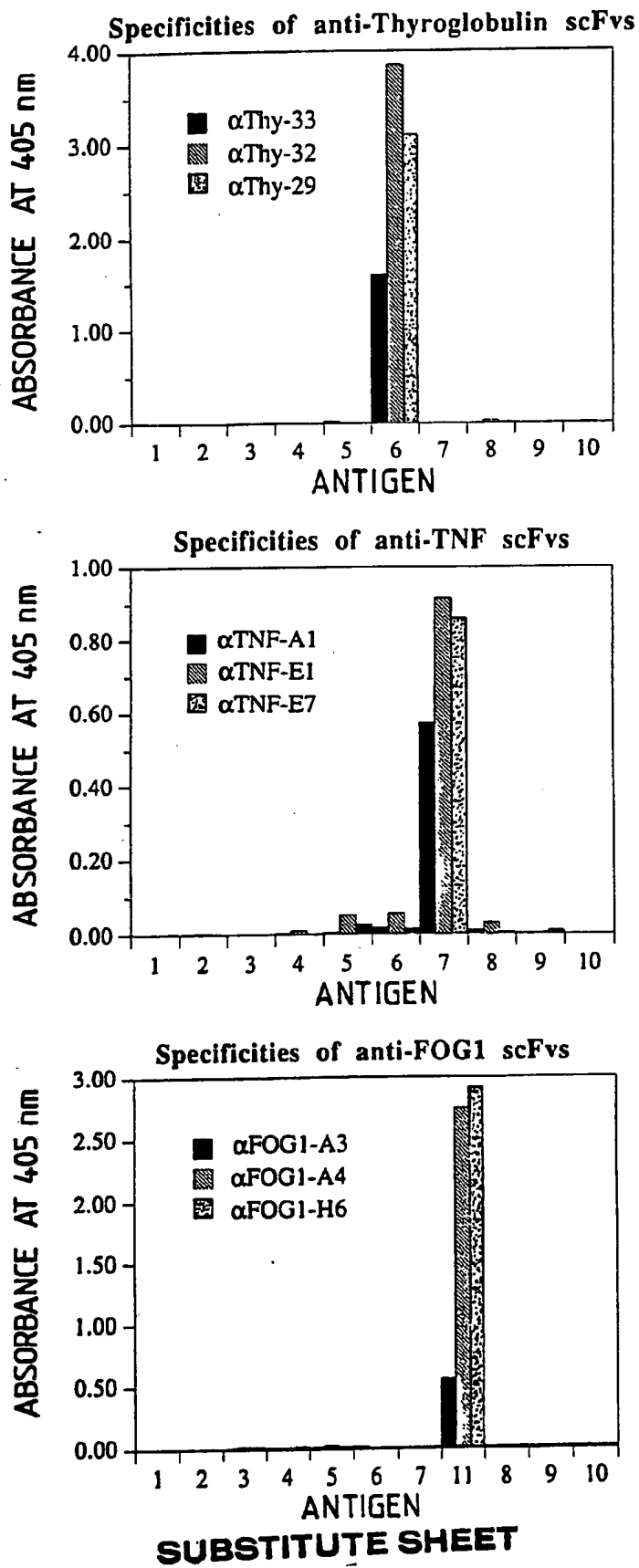
prepared by artificial or synthetic recombination of V-gene sequences.

11. A method according to claim 10 wherein the library is derived from germ line V-gene sequences.

12. A method according to any one of the preceding claims wherein said species of mammal is human.

13. A method according to any one of the preceding claims wherein sbp members selected in (b) displayed at the surface of rgdps are selected or screened to provide an individual rgdp displaying an sbp member or a mixed population of said rgdps, with each rgdp containing nucleic acid encoding the sbp member or a polypeptide chain thereof which is displayed at its surface.

14. A method according to any one of the preceding claims wherein nucleic acid which encodes a selected or screened sbp member and which is derived from an rgdp which displays at its surface a selected or screened sbp member is used to express an sbp member or a fragment or derivative thereof in a recombinant host organism.

15. A method according to claim 14 wherein nucleic acid from one or more rgdps is taken and used to provide encoding nucleic acid in a further method to obtain an individual sbp member or a mixed population of sbp members, or encoding nucleic acid therefor.

16. A method according to claim 14 or claim 15 wherein the expression end product is modified to produce a derivative thereof.

17. A method according to any one of claims 14,15 and 16 wherein the expression end product or derivative thereof is used to prepare a therapeutic or prophylactic medicament or a diagnostic product.

18. Use, in a method according to any one of the preceding claims, of a kit comprising a library of nucleic acid sequences capable of being packaged in rgdps and which encodes a polypeptide chain component part of an antobody for display at the surface of rgdps.

19. Use, in a method according to any one of claims 1 to 17, of a kit comprising a library of rgdps each

containing nucleic acid encoding at least one polypeptide
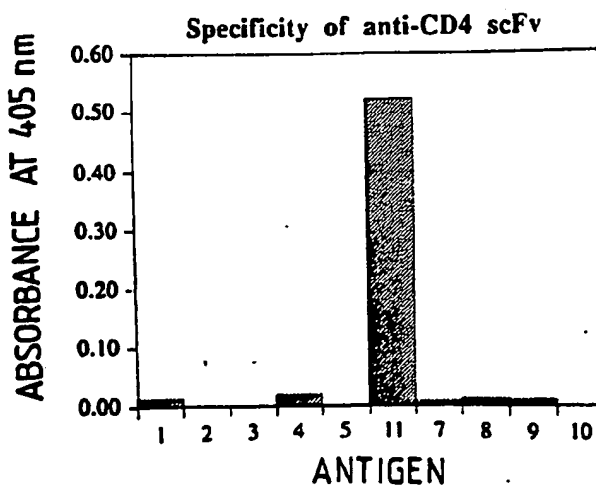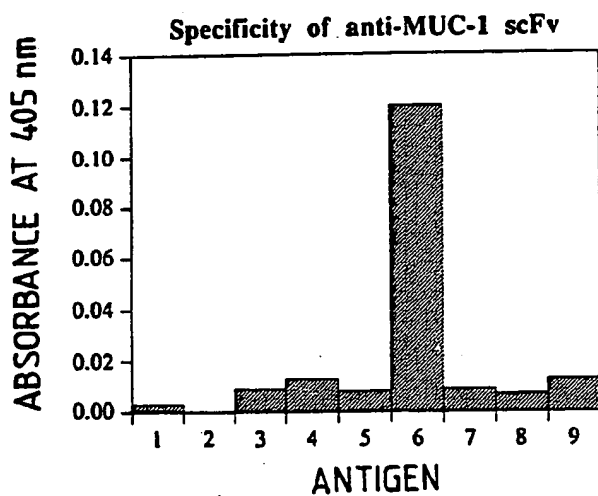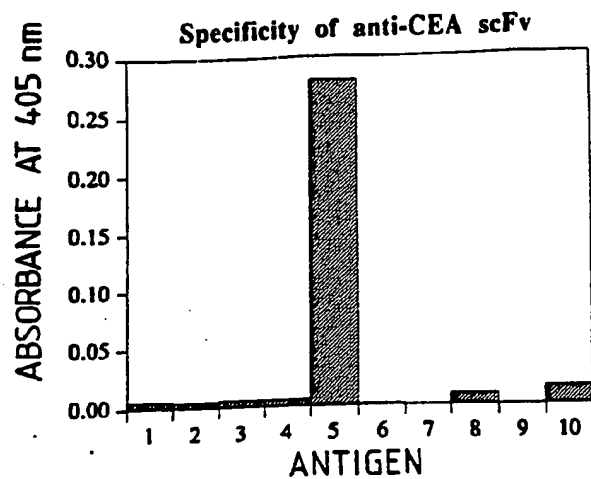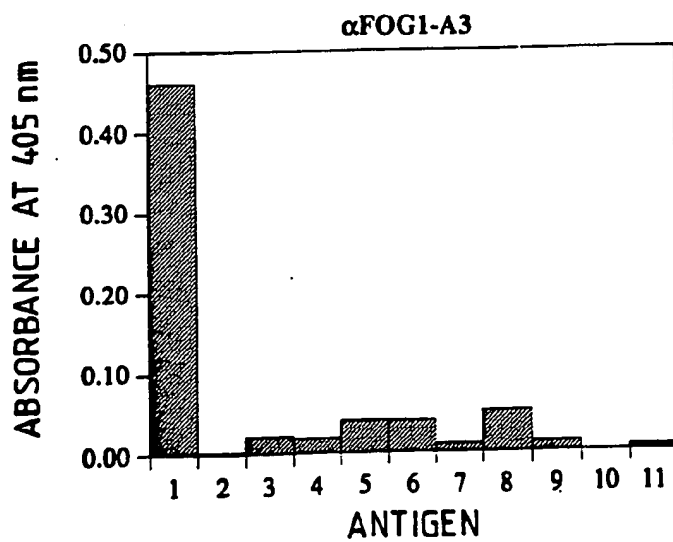chain component part of an antibody.

Fig.1.

Specificities of anti-Thyroglobulin scFvs

Specificities of anti-TNF scFvs

Specificities of anti-FOG1 scFvs

Fig.2.

Specificity of anti-CEA scFv

Specificity of anti-MUC-1 scFv

Specificity of anti-CD4 scFv

Fig.3.
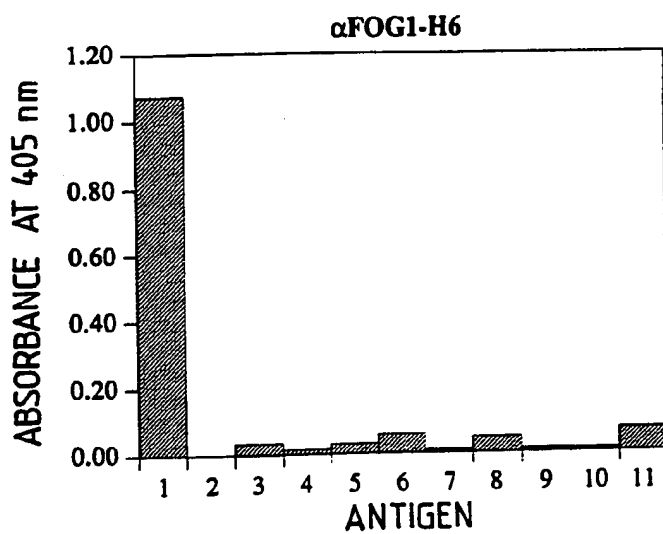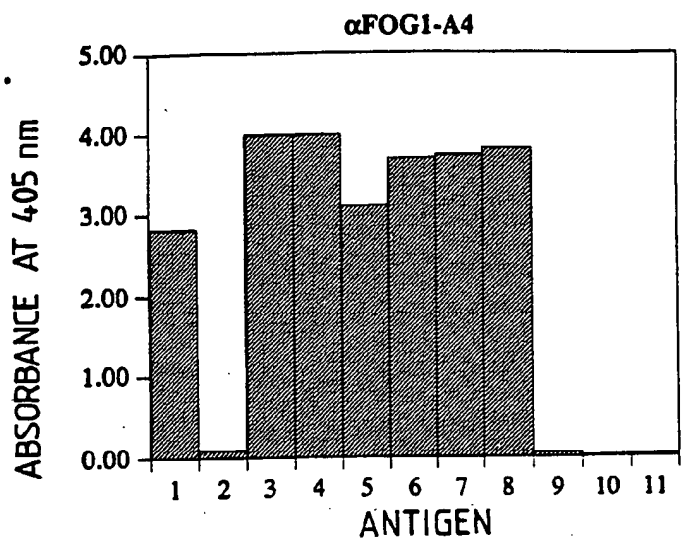
Fig. 4.
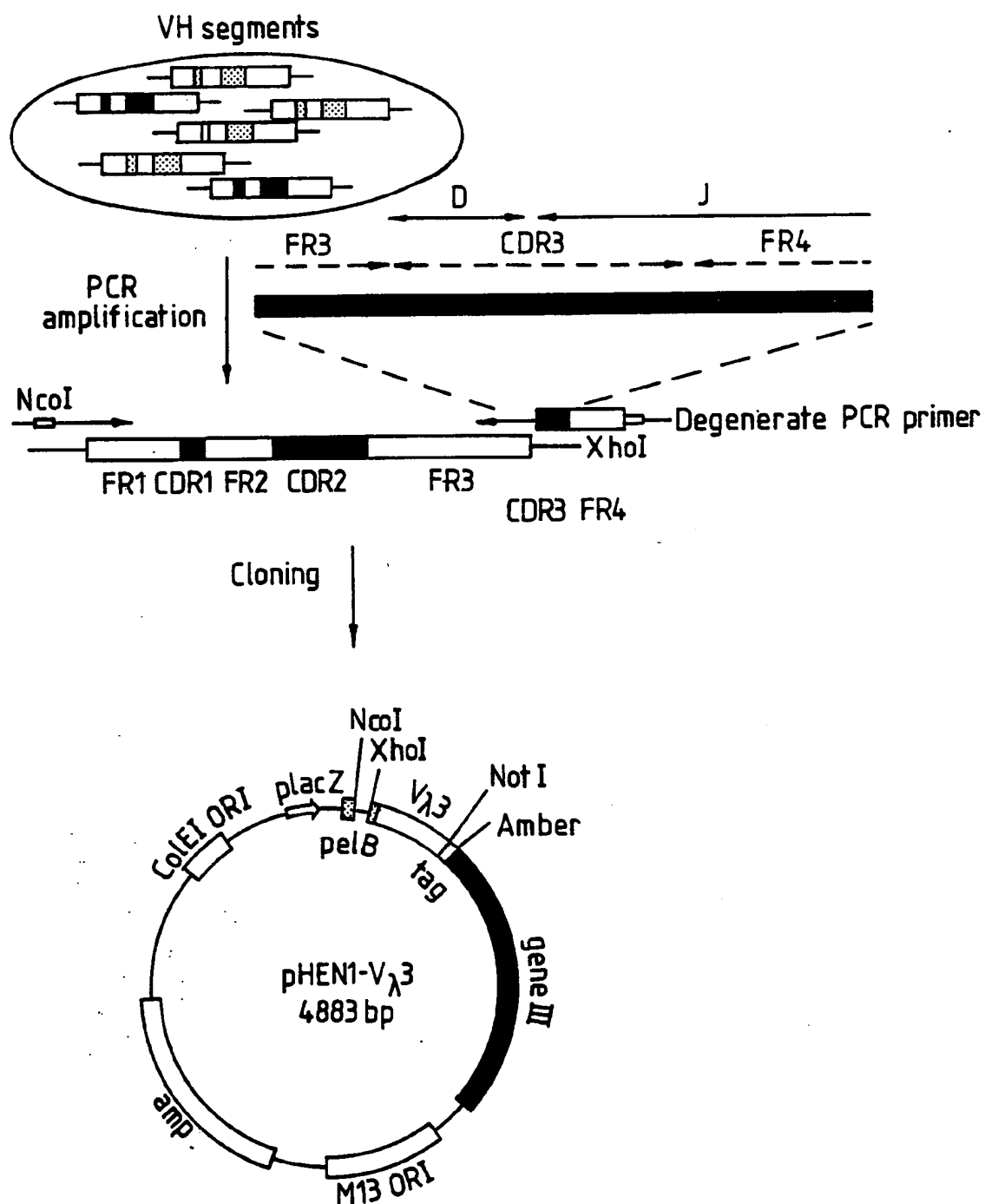
Fig. 5(i)

Fig. 5 (ii)

INTERNATIONAL SEARCH REPORT

PCT/GB 92/02240

International Application No

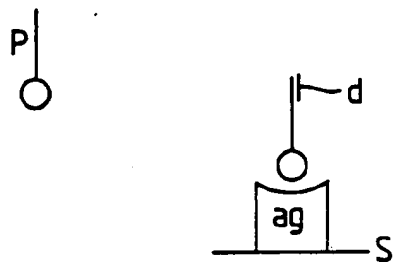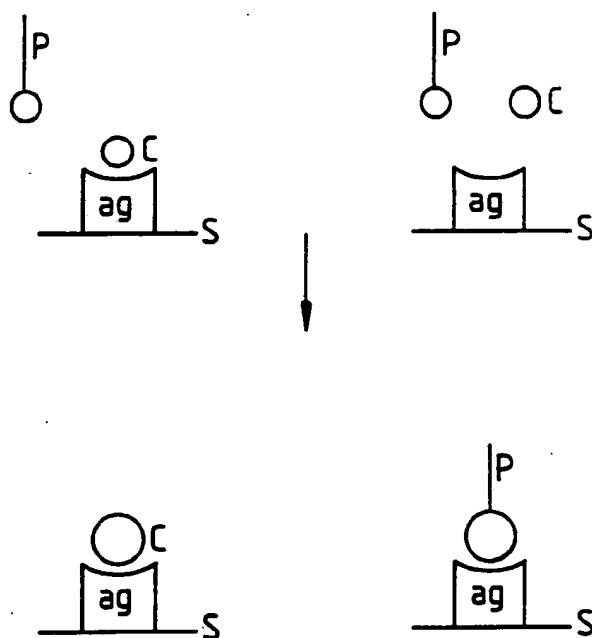## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/13;   C12N15/62;   C07K13/00;   C12P21/08
       G01N33/531;   G01N33/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; G01N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category°	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO,A,9 201 047 (CAMBRIDGE ANTIBODY TECHNOLOGY, LTD, GB) 23 January 1992 cited in the application See Page 11, line 7 - Page 14, line38; Page 16, line 42 - Page 29, lin 37; examples 39-43, 48; the claims ---	1-19
P,X	WO,A,9 220 791 (CAMBRIDGE ANTIBODY TECHNOLOGY LTD, GB) 26 November 1992 see the whole document ---	1-19
X	WO,A,9 014 443 (HUSE, WILLIAM, USA) 29 November 1990 See claims 1, 4, 5, 8-11, 13, 24 ---	1-19

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 MARCH 1993	

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	S.A. NAUCHE

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	WO,A,9 014 430 (SCRIPPS CLINIC AND RESEARCH FOUNDATION, US) 29 November 1990 see the whole document ---	1-19
X	WO,A,9 014 424 (SCRIPPS CLINIC AND RESEARCH FOUNDATION, US) 29 November 1990 see the whole document ---	1-19
X	WO,A,8 806 630 (GENEX CORPORATION, USA) 7 September 1988 see claim 4 ---	6
X	SCIENCE vol. 246, 8 December 1989, LANCASTER, PA pages 1275 - 1281 Huse, W.D. et al.; 'Generation of a large combinatorial library of the Immunoglobulin repertoire in Phage lambda.' see the whole document ---	1-5,8, 12,19
X	NATURE. vol. 349, 24 January 1991, LONDON GB pages 293 - 299 Winter, Greg; Milstein, Cesar; 'Man-made antibodies' cited in the application ---	1-5,10, 11, 13-16,19
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, May 1991, WASHINGTON US pages 4363 - 4366 Kang, A.S. et al.; 'Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surface.' see the whole document ---	1,5,7,8, 12-19
P,X	BIOTECHNOLOGY vol. 10, no. 7, July 1992, NEW YORK US pages 779 - 783 Marks, James D.; Griffiths, Andrew D.; Malmqvist, Magnus; Clackson, Tim P.; Bye, Jacqueline M.; Winter, Greg; 'By-passing immunization: building high affinity human antibodies by chain shuffling' -----	1,6

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB 9202240
SA   67821

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.   17/03/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9201047	23-01-92	AU-A-	8221691	04-02-92
		WO-A-	9220791	26-11-92
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